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(21) International Application Number: PCT/DK98/00444 (22) International Filing Date: 13 October 1998 (13.10.98) (30) Priority Data: 117297 13 October 1997 (13.10.97) DK (71) Applicant: NOVO NORDISK A/S [DK/DK]; Novo Allé, DK-2880 Bagsværd (DK). (72) Inventors: SVEINSEN, Allan, Novo Nordisk a/s, Novo Allé, DK-2880 Bagsværd (DK); BORCHERT, Torben, Vedel; Novo Nordisk a/s, Novo Allé, DK-2880 Bagsværd (DK); BISGÅRD-FRANTZEN, Henrik, Novo Nordisk a/s, Novo Allé, DK-2880 Bagsværd (DK). (74) Common Representative: NOVO NORDISK A/S; Corporate Patents, Novo Allé, DK-2880 Bagsværd (DK).	(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i>	
(54) Title: α -AMYLASE MUTANTS (57) Abstract <p>The invention relates to a variant of a parent Termamyl-like α-amylase, comprising mutations in two, three, four, five or six regions/positions. The variants have increased thermostability at acidic pH and/or at low Ca^{2+} concentrations (relative to the parent). The invention also relates to a DNA construct comprising a DNA sequence encoding an α-amylase variant of the invention, a recombinant expression vector which carries a DNA construct of the invention, a cell which is transformed with a DNA construct of the invention, the use of an α-amylase variant of the invention for washing and/or dishwashing, textile desizing, starch liquefaction, a detergent additive comprising an α-amylase variant of the invention, a manual or automatic dishwashing detergent composition comprising an α-amylase variant of the invention, a method for generating a variant of a parent Termamyl-like α-amylase, which variant exhibits increased thermostability at acidic pH and/or at low Ca^{2+} concentrations (relative to the parent).</p>		

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Title: α -amylase mutants

FIELD OF THE INVENTION

5 The present invention relates, *inter alia*, to novel variants (mutants) of parent Termamyl-like α -amylases, notably variants exhibiting increased thermostability at acidic pH and/or at low Ca^{2+} concentrations (relative to the parent) which are advantageous with respect to applications of the variants in, 10 industrial starch processing particularly (e.g. starch liquefaction or saccharification).

BACKGROUND OF THE INVENTION

α -Amylases (α -1,4-glucan-4-glucanohydrolases, EC 3.2.1.1) 15 constitute a group of enzymes which catalyze hydrolysis of starch and other linear and branched 1,4-glucosidic oligo- and polysaccharides.

There is a very extensive body of patent and scientific literature relating to this industrially very important class of 20 enzymes. A number of α -amylase such as Termamyl-like α -amylases variants are known from e.g. WO 90/11352, WO 95/10603, WO 95/26397, WO 96/23873 and WO 96/23874.

Among more recent disclosures relating to α -amylases, WO 96/23874 provides three-dimensional, X-ray crystal structural 25 data for a Termamyl-like α -amylase which consists of the 300 N-terminal amino acid residues of the *B. amyloliquefaciens* α -amylase and amino acids 301-483 of the C-terminal end of the *B. licheniformis* α -amylase comprising the amino acid sequence (the latter being available commercially under the tradename 30 TermamylTM), and which is thus closely related to the industrially important *Bacillus* α -amylases (which in the present context are embraced within the meaning of the term "Termamyl-like α -amylases", and which include, *inter alia*, the *B. licheniformis*, *B. amyloliquefaciens* and *B. stearothermophilus* 35 α -amylases). WO 96/23874 further describes methodology for

designing, on the basis of an analysis of the structure of a parent Termamyl-like α -amylase, variants of the parent Termamyl-like α -amylase which exhibit altered properties relative to the parent.

5 WO 95/35382 (Gist Brocades B.V.) concerns amylolytic enzymes derived from *B. licheniformis* with improved properties allowing reduction of the Ca^{2+} concentration under application without a loss of performance of the enzyme. The amylolytic enzyme comprises one or more amino acid changes at positions selected
10 from the group of 104, 128, 187, 188 of the *B. licheniformis* α -amylase sequence.

WO 96/23873 (Novo Nordisk) discloses Termamyl-like α -amylase variants which have increased thermostability obtained by pairwise deletion in the region R181*, G182*, T183* and G184* of
15 the sequence shown in SEQ ID NO: 1 herein.

BRIEF DISCLOSURE OF THE INVENTION

The present invention relates to novel α -amylolytic variants (mutants) of a Termamyl-like α -amylase, in particular variants
20 exhibiting increased thermostability (relative to the parent) which are advantageous in connection with the industrial processing of starch (starch liquefaction, saccharification and the like).

The inventors have surprisingly found out that in case of
25 combining two, three, four, five or six mutations (will be described below), the thermostability of Termamyl-like α -amylases is increased at acidic pH and/or at low Ca^{2+} concentration in comparison to single mutations, such as the mutation disclosed in WO 96/23873 (Novo Nordisk), i.e. pairwise
30 deletion in the region R181*, G182*, T183* and G184* of the sequence shown in SEQ ID NO: 1 herein.

The invention further relates to DNA constructs encoding variants of the invention, to composition comprising variants of the invention, to methods for preparing variants of the
35 invention, and to the use of variants and compositions of the invention, alone or in combination with other α -amylolytic

enzymes, in various industrial processes, e.g., starch liquefaction.

BRIEF DESCRIPTION OF THE DRAWING

5 Figure 1 is an alignment of the amino acid sequences of six parent Termamyl-like α -amylases in the context of the invention. The numbers on the Extreme left designate the respective amino acid sequences as follows:

- 1: SEQ ID NO: 2,
- 10 2: Kacamyl,
- 3: SEQ ID NO: 1,
- 4: SEQ ID NO: 5,
- 5: SEQ ID NO: 4,
- 6: SEQ ID NO: 3.

15

DETAILED DISCLOSURE OF THE INVENTION

The Termamyl-like α -amylase

It is well known that a number of α -amylases produced by
20 *Bacillus* spp. are highly homologous on the amino acid level. For instance, the *B. licheniformis* α -amylase comprising the amino acid sequence shown in SEQ ID NO: 4 (commercially available as Termamyl™) has been found to be about 89% homologous with the *B. amyloliquefaciens* α -amylase comprising the amino acid sequence
25 shown in SEQ ID NO: 5 and about 79% homologous with the *B. stearothermophilus* α -amylase comprising the amino acid sequence shown in SEQ ID NO: 3. Further homologous α -amylases include an α -amylase derived from a strain of the *Bacillus* sp. NCIB 12289, NCIB 12512, NCIB 12513 or DSM 9375, all of which are described
30 in detail in WO 95/26397, and the α -amylase described by Tsukamoto et al., Biochemical and Biophysical Research Communications, 151 (1988), pp. 25-31.

Still further homologous α -amylases include the α -amylase produced by the *B. licheniformis* strain described in EP 0252666
35 (ATCC 27811), and the α -amylases identified in WO 91/09353 and

WO 94/18314. Other commercial Termamyl-like *B. licheniformis* α -amylases are Optitherm™ and Takatherm™ (available from Solvay), Maxamyl™ (available from Gist-brocades/Genencor), Spezym AA™ and Spezyme Delta AA™ (available from Genencor), and 5 Keistase™ (available from Daiwa).

Because of the substantial homology found between these α -amylases, they are considered to belong to the same class of α -amylases, namely the class of "Termamyl-like α -amylases".

Accordingly, in the present context, the term "Termamyl-like 10 α -amylase" is intended to indicate an α -amylase which, at the amino acid level, exhibits a substantial homology to Termamyl™, i.e. the *B. licheniformis* α -amylase having the amino acid sequence shown in SEQ ID NO: 4 herein. In other words, a Termamyl-like α -amylase is an α -amylase which has the amino acid 15 sequence shown in SEQ ID NOS: 1, 2, 3, 4, 5, 6, 7 or 8 herein, and the amino acid sequence shown in SEQ ID NO: 1 of WO 95/26397 (the same as the amino acid sequence shown as SEQ ID NO: 7 herein) or in SEQ ID NO: 2 of WO 95/26397 (the same as the amino acid sequence shown as SEQ ID NO: 8 herein) or in Tsukamoto et al., 1988, (which amino acid sequence is shown in SEQ ID NO: 6 20 herein) or i) which displays at least 60%, preferred at least 70%, more preferred at least 75%, even more preferred at least 80%, especially at least 85%, especially preferred at least 90%, even especially more preferred at least 95% homology with at 25 least one of said amino acid sequences shown in SEQ ID NOS 1 or 2 or 3 or 4 or 5 or 6 or 7 or 8 and/or ii) displays immunological cross-reactivity with an antibody raised against at least one of said α -amylases, and/or iii) is encoded by a DNA sequence which hybridizes to the DNA sequences encoding the 30 above-specified α -amylases which are apparent from SEQ ID NOS: 9, 10, 11, or 12 of the present application (which encoding sequences encode the amino acid sequences shown in SEQ ID NOS: 1, 2, 3, 4 and 5 herein, respectively), from SEQ ID NO: 4 of WO 95/26397 (which DNA sequence, together with the stop codon TAA,

is shown in SEQ ID NO: 13 herein and encodes the amino acid sequence shown in SEQ ID NO: 8 herein) and from SEQ ID NO: 5 of WO 95/26397 (shown in SEQ ID NO: 14 herein), respectively.

5 In connection with property i), the "homology" may be determined by use of any conventional algorithm, preferably by use of the GAP programme from the GCG package version 7.3 (June 1993) using default values for GAP penalties, which is a GAP creation penalty of 3.0 and GAP extension penalty of 0.1, (Genetic Computer Group (1991) Programme Manual for the GCG
10 Package, version 7, 575 Science Drive, Madison, Wisconsin, USA 53711).

A structural alignment between Termamyl and a Termamyl-like α -amylase may be used to identify equivalent/corresponding positions in other Termamyl-like α -amylases. One method of
15 obtaining said structural alignment is to use the Pile Up programme from the GCG package using default values of gap penalties, i.e., a gap creation penalty of 3.0 and gap extension penalty of 0.1. Other structural alignment methods include the hydrophobic cluster analysis (Gaboriaud et al., (1987), FEBS
20 LETTERS 224, pp. 149-155) and reverse threading (Huber, T ; Torda, AE, PROTEIN SCIENCE Vol. 7, No. 1 pp. 142-149 (1998)).

Property ii) of the α -amylase, i.e. the immunological cross reactivity, may be assayed using an antibody raised against, or reactive with, at least one epitope of the relevant Termamyl-
25 like α -amylase. The antibody, which may either be monoclonal or polyclonal, may be produced by methods known in the art, e.g. as described by Hudson et al., Practical Immunology, Third edition (1989), Blackwell Scientific Publications. The immunological cross-reactivity may be determined using assays known in the
30 art, examples of which are Western Blotting or radial immunodiffusion assay, e.g. as described by Hudson et al., 1989. In this respect, immunological cross-reactivity between the α -amylases having the amino acid sequences SEQ ID NOS: 1, 2, 3, 4, 5, 6, 7, or 8 respectively, have been found.

35 The oligonucleotide probe used in the characterization of the Termamyl-like α -amylase in accordance with property iii) above

may suitably be prepared on the basis of the full or partial nucleotide or amino acid sequence of the α -amylase in question.

Suitable conditions for testing hybridization involve presoaking in 5xSSC and prehybridizing for 1 hour at -40°C in a solution of 20% formamide, 5xDenhardt's solution, 50mM sodium phosphate, pH 6.8, and 50mg of denatured sonicated calf thymus DNA, followed by hybridization in the same solution supplemented with 100mM ATP for 18 hours at -40°C , followed by three times washing of the filter in 2xSSC, 0.2% SDS at 40°C for 30 minutes (low stringency), preferred at 50°C (medium stringency), more preferably at 65°C (high stringency), even more preferably at -75°C (very high stringency). More details about the hybridization method can be found in Sambrook et al., Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor, 1989.

In the present context, "derived from" is intended not only to indicate an α -amylase produced or producible by a strain of the organism in question, but also an α -amylase encoded by a DNA sequence isolated from such strain and produced in a host organism transformed with said DNA sequence. Finally, the term is intended to indicate an α -amylase which is encoded by a DNA sequence of synthetic and/or cDNA origin and which has the identifying characteristics of the α -amylase in question. The term is also intended to indicate that the parent α -amylase may be a variant of a naturally occurring α -amylase, i.e. a variant which is the result of a modification (insertion, substitution, deletion) of one or more amino acid residues of the naturally occurring α -amylase.

Parent hybrid α -amylases

The parent α -amylase may be a hybrid α -amylase, i.e. an α -amylase which comprises a combination of partial amino acid sequences derived from at least two α -amylases.

The parent hybrid α -amylase may be one which on the basis of amino acid homology and/or immunological cross-reactivity and/or

DNA hybridization (as defined above) can be determined to belong to the Termamyl-like α -amylase family. In this case, the hybrid α -amylase is typically composed of at least one part of a Termamyl-like α -amylase and part(s) of one or more other α -amylases selected from Termamyl-like α -amylases or non-Termamyl-like α -amylases of microbial (bacterial or fungal) and/or mammalian origin.

Thus, the parent hybrid α -amylase may comprise a combination of partial amino acid sequences deriving from at least two Termamyl-like α -amylases, or from at least one Termamyl-like and at least one non-Termamyl-like bacterial α -amylase, or from at least one Termamyl-like and at least one fungal α -amylase. The Termamyl-like α -amylase from which a partial amino acid sequence derives may, e.g., be any of those specific Termamyl-like α -amylases referred to herein.

For instance, the parent α -amylase may comprise a C-terminal part of an α -amylase derived from a strain of *B. licheniformis*, and a N-terminal part of an α -amylase derived from a strain of *B. amyloliquefaciens* or from a strain of *B. stearothermophilus*.

For instance, the parent α -amylase may comprise at least 430 amino acid residues of the C-terminal part of the *B. licheniformis* α -amylase, and may, e.g. comprise a) an amino acid segment corresponding to the 37 N-terminal amino acid residues of the *B. amyloliquefaciens* α -amylase having the amino acid sequence shown in SEQ ID NO: 5 and an amino acid segment corresponding to the 445 C-terminal amino acid residues of the *B. licheniformis* α -amylase having the amino acid sequence shown in SEQ ID No. 4, or b) an amino acid segment corresponding to the 68 N-terminal amino acid residues of the *B. stearothermophilus* α -amylase having the amino acid sequence shown in SEQ ID NO: 3 and an amino acid segment corresponding to the 415 C-terminal amino acid residues of the *B. licheniformis* α -amylase having the amino acid sequence shown in SEQ ID NO: 4.

The non-Termamyl-like α -amylase may, e.g., be a fungal α -amylase, a mammalian or a plant α -amylase or a bacterial α -amylase (different from a Termamyl-like α -amylase). Specific examples of such α -amylases include the *Aspergillus oryzae* TAKA
5 α -amylase, the *A. niger* acid α -amylase, the *Bacillus subtilis* α -amylase, the porcine pancreatic α -amylase and a barley α -amylase. All of these α -amylases have elucidated structures which are markedly different from the structure of a typical Termamyl-like α -amylase as referred to herein.

10 The fungal α -amylases mentioned above, i.e. derived from *A. niger* and *A. oryzae*, are highly homologous on the amino acid level and generally considered to belong to the same family of α -amylases. The fungal α -amylase derived from *Aspergillus oryzae* is commercially available under the tradename Fungamyl™.

15 Furthermore, when a particular variant of a Termamyl-like α -amylase (variant of the invention) is referred to - in a conventional manner - by reference to modification (e.g. deletion or substitution) of specific amino acid residues in the amino acid sequence of a specific Termamyl-like α -amylase, it is
20 to be understood that variants of another Termamyl-like α -amylase modified in the equivalent position(s) (as determined from the best possible amino acid sequence alignment between the respective amino acid sequences) are encompassed thereby.

A preferred embodiment of a variant of the invention is one
25 derived from a *B. licheniformis* α -amylase (as parent Termamyl-like α -amylase), e.g. one of those referred to above, such as the *B. licheniformis* α -amylase having the amino acid sequence shown in SEQ ID NO: 4.

30 Construction of variants of the invention

The construction of the variant of interest may be accomplished by cultivating a microorganism comprising a DNA sequence encoding the variant under conditions which are conducive for producing the variant. The variant may then

subsequently be recovered from the resulting culture broth. This is described in detail further below.

Altered properties of variants of the invention

5 The following discusses the relationship between mutations which may be present in variants of the invention, and desirable alterations in properties (relative to those a parent, Termamyl-like α -amylase) which may result therefrom.

10 Increased thermostability at acidic pH and/or at low Ca^{2+} concentration

Mutations of particular relevance in relation to obtaining variants according to the invention having increased thermostability at acidic pH and/or at low Ca^{2+} concentration
15 include mutations at the following positions (relative to *B. licheniformis* α -amylase, SEQ ID NO: 4):
H156, N172, A181, N188, N190, H205, D207, A209, A210, E211, Q264, N265.

In the context of the invention the term "acidic pH" means a
20 pH below 7.0, especially below the pH range, in which industrial starch liquefaction processes are normally performed, which is between pH 5.5 and 6.2.

In the context of the present invention the term "low Calcium concentration" means concentrations below the normal level used
25 in industrial starch liquefaction. Normal concentrations vary depending of the concentration of free Ca^{2+} in the corn. Normally a dosage corresponding to 1mM (40ppm) is added which together with the level in corn gives between 40 and 60ppm free Ca^{2+} .

In the context of the invention the term "high temperatures"
30 means temperatures between 95°C and 160°C, especially the temperature range in which industrial starch liquefaction processes are normally performed, which is between 95°C and 105°C.

The inventors have now found that the thermostability at
35 acidic pH and/or at low Ca^{2+} concentration may be increased even more by combining certain mutations including the above

mentioned mutations and/or I201 with each other.

Said "certain" mutations are the following (relative to *B. licheniformis* α -amylase, SEQ ID NO: 4):
N190, D207, E211, Q264 and I201.

Said mutation may further be combined with deletions in one, preferably two or even three positions as described in WO 96/23873 (i.e. in positions R181, G182, T183, G184 in SEQ ID NO: 1 herein). According to the invention variants of a parent Termamyl-like α -amylase with α -amylase activity comprising mutations in two, three, four, five or six of the above positions are contemplated.

It should be emphasized that not only the Termamyl-like α -amylases mentioned specifically below are contemplated. Also other commercial Termamyl-like α -amylases are contemplated. An unexhaustive list of such α -amylases is the following:

α -amylases produced by the *B. licheniformis* strain described in EP 0252666 (ATCC 27811), and the α -amylases identified in WO 91/00353 and WO 94/18314. Other commercial Termamyl-like *B. licheniformis* α -amylases are Optitherm™ and Takatherm™ (available from Solvay), Maxamyl™ (available from Gist-brocades/Genencor), Spezym AA™ Spezyme Delta AA™ (available from Genencor), and Keistase™ (available from Daiwa).

It may be mentioned here that amino acid residues, respectively, at positions corresponding to N190, I201, D207 and E211, respectively, in SEQ ID NO: 4 constitute amino acid residues which are conserved in numerous Termamyl-like α -amylases. Thus, for example, the corresponding positions of these residues in the amino acid sequences of a number of Termamyl-like α -amylases which have already been mentioned (vide supra) are as follows:

Table 1.

Termamyl-like α -amylase	N	I	D	E	Q
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	<i>E. licheniformis</i> (SEQ ID NO: 4)	N190	I201	D207	E211	Q264
	<i>B. amyloliquefaciens</i> (SEQ ID NO: 5)	N190	V201	D207	E211	Q264
	<i>B. stearothermophilus</i> (SEQ ID NO: 3)	N193	I204	E210	E214	---
	<i>Bacillus</i> WO 95/26397 (SEQ ID NO: 2)	N195	V206	E212	E216	---
5	<i>Bacillus</i> WO 95/26397 (SEQ ID NO: 1)	N195	V206	E213	E216	---
	" <i>Bacillus</i> sp. #707" (SEQ ID NO: 6)	N195	I206	E212	E216	---

Mutations of these conserved amino acid residues are very important in relation to improving thermostability at acidic pH and/or at low calcium concentration, and the following mutations are of particular interest in this connection (with reference to the numbering of the *B. licheniformis* amino acid sequence shown in SEQ ID NO: 4).

Pair-wise amino acid deletions at positions corresponding to R179-G182 in SEQ ID NO: 5 corresponding to a gap in Seq ID NO: 4, when aligned with a numerous Termamyl-like α -amylases. Thus, for example, the corresponding positions of these residues in the amino acid sequences of a number of Termamyl-like α -amylases which have already been mentioned (*vide supra*) are as follows:

Table 2.

	Termamyl-like α -amylase	Pair wise amino acid deletions among
25	<i>B. amyloliquefaciens</i> (SEQ ID No.5)	R176, G177, E178, G179
	<i>B. stearothermophilus</i> (SEQ ID No.3)	R179, G180, I181, G182
	<i>Bacillus</i> WO 95/26397 (SEQ ID No.2)	R181, G182, T183, G184
	<i>Bacillus</i> WO 95/26397 (SEQ ID No.1)	R181, G182, D183, G184
30	" <i>Bacillus</i> sp. #707" (SEQ ID No.6)	E181, G182, H183, G184

When using SEQ ID NO: 1 to SEQ ID NO: 6 as the backbone (i.e. as the parent Termamyl-like α -amylase) two, three, four, five or six mutations may according to the invention be made in the following regions/positions to increase the thermostability at acidic pH and/or at low Ca^{2+} concentrations (relative to the parent):

(relative to Seq ID NO: 1 herein):

- 1: R181*, G182*, T183*, G184*
2: N195A,R,D,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
3: V206A,R,D,N,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
4: E212A,R,D,N,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
5 5: E216A,R,D,N,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
6: K269A,R,D,N,C,E,Q,G,H,I,L,M,F,P,S,T,W,Y,V;
(relative to SEQ ID NO: 2 herein):
1: R181*,G182*,D183*,G184*
2: N195A,R,D,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
10 3: V206A,R,D,N,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
4: E212A,R,D,N,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
5: E216A,R,D,N,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
6: K269A,R,D,N,C,E,Q,G,H,I,L,M,F,P,S,T,W,Y,V;
(Relative to SEQ ID NO: 3 herein):
15 1: R179*,G180,I181*,G182*
2: N193A,R,D,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
3: L204A,R,D,N,C,E,Q,G,H,I,K,M,F,P,S,T,W,Y,V;
4: E210A,R,D,N,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
5: E214A,R,D,N,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
20 6: S267A,R,D,N,C,E,Q,G,H,I,L,K,M,F,P,T,W,Y,V
Relative to SEQ ID NO: 4 herein):
1: Q178*,G179*
2: N190A,R,D,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
3: I201A,R,D,N,C,E,Q,G,H,L,K,M,F,P,S,T,W,Y,V;
25 4: D207A,R,N,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
5: E211A,R,D,N,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
6: Q264A,R,D,N,C,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
(relative to SEQ ID NO: 5 herein):
1: R176*,G177*,E178,G179*
30 2: N190A,R,D,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
3: V201A,R,D,N,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
4: D207A,R,N,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
5: E211A,R,D,N,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
6: Q264A,R,D,N,C,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
35 (relative to SEQ ID NO: 6 herein):
1: R181*,G182*,H183*,G184*
2: N195A,R,D,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;

- 3: I206A, R, D, N, C, E, Q, G, H, I, L, K, M, F, P, S, T, W, Y, V;
4: E212A, R, D, N, C, Q, G, H, I, L, K, M, F, P, S, T, W, Y, V;
5: E216A, R, D, N, C, Q, G, H, I, L, K, M, F, P, S, T, W, Y, V;
6: K269A, R, D, N, C, E, Q, G, H, I, L, M, F, P, S, T, W, Y, V.

5 Contemplated according to the present invention is combining three, four, five or six mutation.

Specific double mutations for backbone SEQ ID NO: 1 to SEQ ID NO: 6 are listed in the following.

Using SEQ ID NO: 1 as the backbone the following double
10 mutations resulting in the desired effect are contemplated according to the invention:

- R181*/G182*/N195A, R, D, C, E, Q, G, H, I, L, K, M, F, P, S, T, W, Y, V;
-G182*/T183*/N195A, R, D, C, E, Q, G, H, I, L, K, M, F, P, S, T, W, Y, V;
-T183*/G184*/N195A, R, D, C, E, Q, G, H, I, L, K, M, F, P, S, T, W, Y, V;
15 -R181*/G182*/V206A, R, D, N, C, E, Q, G, H, I, L, K, M, F, P, S, T, W, Y;
-G182*/T183*/V206A, R, D, N, C, E, Q, G, H, I, L, K, M, F, P, S, T, W, Y;
-T183*/G184*/V206A, R, D, N, C, E, Q, G, H, I, L, K, M, F, P, S, T, W, Y;
-R181*/G182*/E212A, R, D, N, C, Q, G, H, I, L, K, M, F, P, S, T, W, Y, V;
-G182*/T183*/E212A, R, D, N, C, Q, G, H, I, L, K, M, F, P, S, T, W, Y, V;
20 -T183*/G184*/E212A, R, D, N, C, Q, G, H, I, L, K, M, F, P, S, T, W, Y, V;
-R181*/G182*/E216A, R, D, N, C, Q, G, H, I, L, K, M, F, P, S, T, W, Y, V;
-G182*/T183*/E216A, R, D, N, C, Q, G, H, I, L, K, M, F, P, S, T, W, Y, V;
-T183*/G184*/E216A, R, D, N, C, Q, G, H, I, L, K, M, F, P, S, T, W, Y, V;
-R181*/G182*/K269A, R, D, N, C, E, Q, G, H, I, L, M, F, P, S, T, W, Y, V;
25 -G182*/T183*/K269A, R, D, N, C, E, Q, G, H, I, L, M, F, P, S, T, W, Y, V;
-T183*/G184*/K269A, R, D, N, C, E, Q, G, H, I, L, M, F, P, S, T, W, Y, V;
-N195A, R, D, C, E, Q, G, H, I, L, K, M, F, P, S, T, W, Y, V
/V206A, R, D, N, C, E, Q, G, H, I, L, K, M, F, P, S, T, W, Y;
-N195A, R, D, C, E, Q, G, H, I, L, K, M, F, P, S, T, W, Y, V
30 /E212A, R, D, N, C, Q, G, H, I, L, K, M, F, P, S, T, W, Y, V;
-N195A, R, D, C, E, Q, G, H, I, L, K, M, F, P, S, T, W, Y, V
/E216A, R, D, N, C, Q, G, H, I, L, K, M, F, P, S, T, W, Y, V;
-N195A, R, D, C, E, Q, G, H, I, L, K, M, F, P, S, T, W, Y, V
/K269A, R, D, N, C, E, Q, G, H, I, L, M, F, P, S, T, W, Y, V;
35 -V206A, R, D, N, C, E, Q, G, H, I, L, K, M, F, P, S, T, W, Y
/E212A, R, D, N, C, Q, G, H, I, L, K, M, F, P, S, T, W, Y, V;
-V206A, R, D, N, C, E, Q, G, H, I, L, K, M, F, P, S, T, W, Y
/E216A, R, D, N, C, Q, G, H, I, L, K, M, F, P, S, T, W, Y, V;

-V206A, R, D, N, C, E, Q, G, H, I, L, K, M, F, P, S, T, W, Y
/K269A, R, D, N, C, E, Q, G, H, I, L, M, F, P, S, T, W, Y, V;
-E212A, R, D, N, C, Q, G, H, I, L, K, M, F, P, S, T, W, Y, V
/E216A, R, D, N, C, Q, G, H, I, L, K, M, F, P, S, T, W, Y, V;
5 E212A, R, D, N, C, Q, G, H, I, L, K, M, F, P, S, T, W, Y, V
/K269A, R, D, N, C, E, Q, G, H, I, L, M, F, P, S, T, W, Y, V;
-E216A, R, D, N, C, Q, G, H, I, L, K, M, F, P, S, T, W, Y, V
/K269A, R, D, N, C, E, Q, G, H, I, L, M, F, P, S, T, W, Y, V;

Using SEQ ID NO: 2 as the backbone the following double
10 mutations resulting in the desired effect are contemplated
according to the invention:

-R181*/G182*/N195A, R, D, C, E, Q, G, H, I, L, K, M, F, P, S, T, W, Y, V;
-G182*/D183*/N195A, R, D, C, E, Q, G, H, I, L, K, M, F, P, S, T, W, Y, V;
-D183*/G184*/N195A, R, D, C, E, Q, G, H, I, L, K, M, F, P, S, T, W, Y, V;
15 -R181*/G182*/V206A, R, D, N, C, E, Q, G, H, I, L, K, M, F, P, S, T, W, Y;
-G182*/T183*/V206A, R, D, N, C, E, Q, G, H, I, L, K, M, F, P, S, T, W, Y;
-T183*/G184*/V206A, R, D, N, C, E, Q, G, H, I, L, K, M, F, P, S, T, W, Y;
-R181*/G182*/E212A, R, D, N, C, Q, G, H, I, L, K, M, F, P, S, T, W, Y, V;
-G182*/T183*/E212A, R, D, N, C, Q, G, H, I, L, K, M, F, P, S, T, W, Y, V;
20 -T183*/G184*/E212A, R, D, N, C, Q, G, H, I, L, K, M, F, P, S, T, W, Y, V;
-R181*/G182*/E216A, R, D, N, C, Q, G, H, I, L, K, M, F, P, S, T, W, Y, V;
-G182*/T183*/E216A, R, D, N, C, Q, G, H, I, L, K, M, F, P, S, T, W, Y, V;
-T183*/G184*/E216A, R, D, N, C, Q, G, H, I, L, K, M, F, P, S, T, W, Y, V;
-R181*/G182*/K269A, R, D, N, C, E, Q, G, H, I, L, M, F, P, S, T, W, Y, V;
25 -G182*/T183*/K269A, R, D, N, C, E, Q, G, H, I, L, M, F, P, S, T, W, Y, V;
-T183*/G184*/K269A, R, D, N, C, E, Q, G, H, I, L, M, F, P, S, T, W, Y, V;
-N195 A, R, D, C, E, Q, G, H, I, L, K, M, F, P, S, T, W, Y, V
/V206A, R, D, N, C, E, Q, G, H, I, L, K, M, F, P, S, T, W, Y;
-N195 A, R, D, C, E, Q, G, H, I, L, K, M, F, P, S, T, W, Y, V
30 /E212A, R, D, N, C, Q, G, H, I, L, K, M, F, P, S, T, W, Y, V;
-N195A, R, D, C, E, Q, G, H, I, L, K, M, F, P, S, T, W, Y, V
/E216A, R, D, N, C, Q, G, H, I, L, K, M, F, P, S, T, W, Y, V;
-N195A, R, D, C, E, Q, G, H, I, L, K, M, F, P, S, T, W, Y, V
/K269A, R, D, N, C, E, Q, G, H, I, L, M, F, P, S, T, W, Y, V;
35 -V206A, R, D, N, C, E, Q, G, H, I, L, K, M, F, P, S, T, W, Y
/E212A, R, D, N, C, Q, G, H, I, L, K, M, F, P, S, T, W, Y, V;
-V206 A, R, D, N, C, E, Q, G, H, I, L, K, M, F, P, S, T, W, Y

/E216A,R,D,N,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
-V206A,R,D,N,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y
/K269A,R,D,N,C,E,Q,G,H,I,L,M,F,P,S,T,W,Y,V;
-E212A,R,D,N,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V
5 /E216A,R,D,N,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
-E212A,R,D,N,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V
/K269A,R,D,N,C,E,Q,G,H,I,L,M,F,P,S,T,W,Y,V;
-E216A,R,D,N,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V
/K269A,R,D,N,C,E,Q,G,H,I,L,M,F,P,S,T,W,Y,V;

10 Using SEQ ID NO. 3 as the backbone the following double mutations resulting in the desired effect are contemplated according to the invention:

-R179*/G180*/N193A,R,D,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
-G180*/I181*/N193A,R,D,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
15 -I181*/G182*/N193A,R,D,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
-R179*/G180*/L204A,R,D,N,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
-G180*/I181*/L204A,R,D,N,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
-I181*/G182*/L204A,R,D,N,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
-R179*/G180*/E210A,R,D,N,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
20 -G180*/I181*/E210A,R,D,N,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
-I181*/G182*/E210A,R,D,N,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
-R179*/G180*/E214A,R,D,N,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
-G180*/I181*/E214A,R,D,N,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
-I181*/G182*/E214A,R,D,N,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
25 -R179*/G180*/S267A,R,D,N,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
-G180*/I181*/S267A,R,D,N,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
-I181*/G182*/S267A,R,D,N,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
-N193A,R,D,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V
/L204A,R,D,N,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
30 -N193A,R,D,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V
/E210A,R,D,N,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
-N193A,R,D,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V
/E214A,R,D,N,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
-N193A,R,D,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V
35 /S267A,R,D,N,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
-L204A,R,D,N,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V
/E210A,R,D,N,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;

-L204A,R,D,N,C,E,Q,G,H,I,K,M,F,P,S,T,W,Y,V
 /E214A,R,D,N,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
 -L204A,R,D,N,C,E,Q,G,H,I,K,M,F,P,S,T,W,Y,V
 /S267A,R,D,N,C,E,Q,G,H,I,L,K,M,F,P,T,W,Y,V;
 5 -E210A,R,D,N,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V
 /E216A,R,D,N,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
 -E210A,R,D,N,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V
 /S267A,R,D,N,C,E,Q,G,H,I,L,K,M,F,P,T,W,Y,V;
 -E214A,R,D,N,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V
 10 /S267A,R,D,N,C,E,Q,G,H,I,L,K,M,F,P,T,W,Y,V;

Using SEQ ID NO. 4 as the backbone the following double mutations resulting in the desired effect are contemplated according to the invention:

-Q178*/G179*/N190A,R,D,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
 15 -Q178*/G179*/I201A,R,D,N,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
 -Q178*/G179*/D207A,R,N,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
 -Q178*/G179*/E211A,R,D,N,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
 -R179*/G180*/Q264A,R,D,N,C,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
 -N190/I201A,R,D,N,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
 20 -N190/D207A,R,N,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
 -N190/E211A,R,D,N,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
 -N190/Q264A,R,D,N,C,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
 -I201/D207A,R,N,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
 -I201/E211A,R,D,N,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
 25 -I201/Q264A,R,D,N,C,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
 -D207/E211A,R,D,N,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
 -D207/Q264A,R,D,N,C,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
 -E211/Q264A,R,D,N,C,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;

Using SEQ ID NO: 5 as the backbone the following double mutations resulting in the desired effect are contemplated according to the invention:

-R176*/G177*/N190A,R,D,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
 -G177*/E178*/N190A,R,D,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
 -E178*/G179*/N190A,R,D,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
 35 -R176*/G177*/V201A,R,D,N,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y;
 -G176*/E178*/V201A,R,D,N,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y;
 -E178*/G179*/V201A,R,D,N,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y;

-R176*/G177*/D207A,R,N,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
-G177*/E178*/D207A,R,N,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
-E178*/G179*/D207A,R,N,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
-R176*/G177*/E211A,R,D,N,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
5 -G177*/E178*/E211A,R,D,N,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
-E178*/G179*/E211A,R,D,N,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
-R176*/G177*/Q264A,R,D,N,C,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
-G177*/E178*/Q264A,R,D,N,C,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
-E178*/G179*/Q264A,R,D,N,C,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
10 -N190A,R,D,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V
/V201A,R,D,N,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y;
-N190A,R,D,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V
/D207A,R,N,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
-N190A,R,D,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V
15 /E211A,R,D,N,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
-N190A,R,D,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V
/Q264A,R,D,N,C,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
-V201A,R,D,N,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y
/D207A,R,N,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
20 -V201A,R,D,N,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y
/E211A,R,D,N,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
-V201A,R,D,N,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y
/Q264A,R,D,N,C,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
-D207A,R,N,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V
25 /E211A,R,D,N,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
-D207A,R,N,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V
/Q264A,R,D,N,C,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
-E211A,R,D,N,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V
/Q264A,R,D,N,C,E,G,H,I,L,K,M,F,P,S,T,W,Y,V.

30 Using SEQ ID NO: 6 as the backbone the following double
mutations resulting in the desired effect are contemplated
according to the invention:

-R181*/G182*/N195A,R,D,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
-G182*/H183*/N195A,R,D,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
35 -H183*/G184*/N195A,R,D,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
-R181*/G182*/I206A,R,D,N,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
-G182*/H183*/I206A,R,D,N,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;

-H183*/G184*/I206A,R,D,N,C,E,Q,G,H,L,K,M,F,P,S,T,W,Y,V;
 -R181*/G182*/E212A,R,D,N,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
 -G182*/H183*/E212A,R,D,N,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
 -H183*/G184*/E212A,R,D,N,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
 5 -R181*/G182*/E216A,R,D,N,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
 -G182*/H183*/E216A,R,D,N,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
 -H183*/G184*/E216A,R,D,N,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
 -R181*/G182*/K269A,R,D,N,C,E,Q,G,H,I,L,M,F,P,S,T,W,Y,V;
 -G182*/H183*/K269A,R,D,N,C,E,Q,G,H,I,L,M,F,P,S,T,W,Y,V;
 10 -H183*/G184*/K269A,R,D,N,C,E,Q,G,H,I,L,M,F,P,S,T,W,Y,V;
 -N195A,R,D,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V
 /I206A,R,D,N,C,E,Q,G,H,L,K,M,F,P,S,T,W,Y,V;
 -N195A,R,D,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V
 /E212A,R,D,N,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
 15 -N195A,R,D,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V
 /E216A,R,D,N,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
 -N195A,R,D,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V
 /K269A,R,D,N,C,E,Q,G,H,I,L,M,F,P,S,T,W,Y,V;
 -I206A,R,D,N,C,E,Q,G,H,L,K,M,F,P,S,T,W,Y,V
 20 /E212A,R,D,N,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
 -I206A,R,D,N,C,E,Q,G,H,L,K,M,F,P,S,T,W,Y,V
 /E216A,R,D,N,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
 -I206A,R,D,N,C,E,Q,G,H,L,K,M,F,P,S,T,W,Y,V
 /K269A,R,D,N,C,E,Q,G,H,I,L,M,F,P,S,T,W,Y,V;
 25 -E212A,R,D,N,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V
 /E216A,R,D,N,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
 -E212A,R,D,N,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V
 /K269A,R,D,N,C,E,Q,G,H,I,L,M,F,P,S,T,W,Y,V;
 -E216A,R,D,N,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V
 30 /K269A,R,D,N,C,E,Q,G,H,I,L,M,F,P,S,T,W,Y,V;

All Termamyl-like α -amylase defined above may suitably be used as backbone for preparing variants of the invention.

However, in a preferred embodiment the variant comprises the following mutations: N190F/Q264S in SEQ ID NO: 4 or in
 35 corresponding positions in another parent Termamyl-like α -amylases.

In another embodiment the variant of the invention comprises

the following mutations: I181*/G182*/N193F in SEQ ID NO: 3 (TVB146) or in corresponding positions in another parent Termamyl-like α -amylases. Said variant may further comprise a substitution in position E214Q.

5 In a preferred embodiment of the invention the parent Termamyl-like α -amylase is a hybrid α -amylase of SEQ ID NO: 4 and SEQ ID NO: 5. Specifically, the parent hybrid Termamyl-like α -amylase may be a hybrid alpha-amylase comprising the 445 C-terminal amino acid residues of the *B. licheniformis* α -amylase
10 shown in SEQ ID NO: 4 and the 37 N-terminal amino acid residues of the α -amylase derived from *B. amyloliquefaciens* shown in SEQ ID NO: 5, which may suitably further have the following mutations: H156Y+A181T+N190F+A209V+Q264S (using the numbering in SEQ ID NO: 4). The latter mentioned hybrid is used in the
15 examples below and is referred to as LE174.

General mutations in variants of the invention

It may be preferred that a variant of the invention comprises one or more modifications in addition to those outlined above.

20 Thus, it may be advantageous that one or more proline residues present in the part of the α -amylase variant which is modified is/are replaced with a non-proline residue which may be any of the possible, naturally occurring non-proline residues, and which preferably is an alanine, glycine, serine, threonine,
25 valine or leucine.

Analogously, it may be preferred that one or more cysteine residues present among the amino acid residues with which the parent α -amylase is modified is/are replaced with a non-cysteine residue such as serine, alanine, threonine, glycine, valine or
30 leucine.

Furthermore, a variant of the invention may - either as the only modification or in combination with any of the above outlined modifications - be modified so that one or more Asp and/or Glu present in an amino acid fragment corresponding to
35 the amino acid fragment 185-209 of SEQ ID NO: 4 is replaced by an Asn and/or Gln, respectively. Also of interest is the

replacement, in the Termamyl-like α -amylase, of one or more of the Lys residues present in an amino acid fragment corresponding to the amino acid fragment 185-209 of SEQ ID NO: 4 by an Arg.

It will be understood that the present invention encompasses 5 variants incorporating two or more of the above outlined modifications.

Furthermore, it may be advantageous to introduce point-mutations in any of the variants described herein.

10 Methods for preparing α -amylase variants

Several methods for introducing mutations into genes are known in the art. After a brief discussion of the cloning of α -amylase-encoding DNA sequences, methods for generating mutations at specific sites within the α -amylase-encoding sequence will be 15 discussed.

Cloning a DNA sequence encoding an α -amylase

The DNA sequence encoding a parent α -amylase may be isolated from any cell or microorganism producing the α -amylase in question, using various methods well known in the art. First, a 20 genomic DNA and/or cDNA library should be constructed using chromosomal DNA or messenger RNA from the organism that produces the α -amylase to be studied. Then, if the amino acid sequence of the α -amylase is known, homologous, labelled oligonucleotide 25 probes may be synthesized and used to identify α -amylase-encoding clones from a genomic library prepared from the organism in question. Alternatively, a labelled oligonucleotide probe containing sequences homologous to a known α -amylase gene could be used as a probe to identify α -amylase-encoding clones, 30 using hybridization and washing conditions of lower stringency.

Yet another method for identifying α -amylase-encoding clones would involve inserting fragments of genomic DNA into an expression vector, such as a plasmid, transforming α -amylase-negative bacteria with the resulting genomic DNA library, and

then plating the transformed bacteria onto agar containing a substrate for α -amylase, thereby allowing clones expressing the α -amylase to be identified.

Alternatively, the DNA sequence encoding the enzyme may be prepared synthetically by established standard methods, e.g. the phosphoroamidite method described by S.L. Beaucage and M.H. Caruthers (1981) or the method described by Matthes et al. (1984). In the phosphoroamidite method, oligonucleotides are synthesized, e.g. in an automatic DNA synthesizer, purified, annealed, ligated and cloned in appropriate vectors.

Finally, the DNA sequence may be of mixed genomic and synthetic origin, mixed synthetic and cDNA origin or mixed genomic and cDNA origin, prepared by ligating fragments of synthetic, genomic or cDNA origin (as appropriate, the fragments corresponding to various parts of the entire DNA sequence), in accordance with standard techniques. The DNA sequence may also be prepared by polymerase chain reaction (PCR) using specific primers, for instance as described in US 4,683,202 or R.K. Saiki et al. (1988).

Site-directed mutagenesis

Once an α -amylase-encoding DNA sequence has been isolated, and desirable sites for mutation identified, mutations may be introduced using synthetic oligonucleotides. These oligonucleotides contain nucleotide sequences flanking the desired mutation sites; mutant nucleotides are inserted during oligonucleotide synthesis. In a specific method, a single-stranded gap of DNA, bridging the α -amylase-encoding sequence, is created in a vector carrying the α -amylase gene. Then the synthetic nucleotide, bearing the desired mutation, is annealed to a homologous portion of the single-stranded DNA. The remaining gap is then filled in with DNA polymerase I (Klenow fragment) and the construct is ligated using T4 ligase. A specific example of this method is described in Morinaga et al. (1984). US 4,760,025 discloses the introduction of oligonucleotides encoding multiple

mutations by performing minor alterations of the cassette. However, an even greater variety of mutations can be introduced at any one time by the Morinaga method, because a multitude of oligonucleotides, of various lengths, can be introduced.

- 5 Another method for introducing mutations into α -amylase-encoding DNA sequences is described in Nelson and Long (1989). It involves the 3-step generation of a PCR fragment containing the desired mutation introduced by using a chemically synthesized DNA strand as one of the primers in the PCR reactions.
- 10 From the PCR-generated fragment, a DNA fragment carrying the mutation may be isolated by cleavage with restriction endonucleases and reinserted into an expression plasmid.

Random Mutagenesis

- 15 Random mutagenesis is suitably performed either as localised or region-specific random mutagenesis in at least three parts of the gene translating to the amino acid sequence shown in question, or within the whole gene.

- 20 The random mutagenesis of a DNA sequence encoding a parent α -amylase may be conveniently performed by use of any method known in the art.

- In relation to the above, a further aspect of the present invention relates to a method for generating a variant of a
- 25 parent α -amylase, e.g. wherein the variant exhibits altered or increased thermal stability relative to the parent, the method comprising:

- (a) subjecting a DNA sequence encoding the parent α -amylase to random mutagenesis,
- 30 (b) expressing the mutated DNA sequence obtained in step (a) in a host cell, and
- (c) screening for host cells expressing an α -amylase variant which has an altered property (i.e. thermal stability) relative to the parent α -amylase.

Step (a) of the above method of the invention is preferably performed using doped primers.

For instance, the random mutagenesis may be performed by use of a suitable physical or chemical mutagenizing agent, by use of a suitable oligonucleotide, or by subjecting the DNA sequence to PCR generated mutagenesis. Furthermore, the random mutagenesis may be performed by use of any combination of these mutagenizing agents. The mutagenizing agent may, e.g., be one which induces transitions, transversions, inversions, scrambling, deletions, and/or insertions.

Examples of a physical or chemical mutagenizing agent suitable for the present purpose include ultraviolet (UV) ir-radiation, hydroxylamine, N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), O-methyl hydroxylamine, nitrous acid, ethyl methane sulphonate (EMS), sodium bisulphite, formic acid, and nucleotide analogues. When such agents are used, the mutagenesis is typically performed by incubating the DNA sequence encoding the parent enzyme to be mutagenized in the presence of the mutagenizing agent of choice under suitable conditions for the mutagenesis to take place, and selecting for mutated DNA having the desired properties.

When the mutagenesis is performed by the use of an oligonucleotide, the oligonucleotide may be doped or spiked with the three non-parent nucleotides during the synthesis of the oligonucleotide at the positions which are to be changed. The doping or spiking may be done so that codons for unwanted amino acids are avoided. The doped or spiked oligonucleotide can be incorporated into the DNA encoding the α -amylase enzyme by any published technique, using e.g. PCR, LCR or any DNA polymerase and ligase as deemed appropriate.

Preferably, the doping is carried out using "constant random doping", in which the percentage of wild-type and mutation in each position is predefined. Furthermore, the doping may be directed toward a preference for the introduction of certain nucleotides, and thereby a preference for the introduction of one or more specific amino acid residues. The doping may be made, e.g., so as to allow for the introduction of 90% wild type and 10% mutations in each position. An additional consideration in the choice of a doping scheme is based on genetic as well as protein-structural constraints. The doping scheme may be made by using the DOPE program which, *inter alia*, ensures that introduction of stop codons is avoided.

When PCR-generated mutagenesis is used, either a chemically treated or non-treated gene encoding a parent α -amylase is subjected to PCR under conditions that increase the mis-incorporation of nucleotides (Deshler 1992; Leung et al., Technique, Vol.1, 1989, pp. 11-15).

A mutator strain of *E. coli* (Fowler et al., Molec. Gen. Genet., 133, 1974, pp. 179-191), *S. cerevisiae* or any other microbial organism may be used for the random mutagenesis of the DNA encoding the α -amylase by, e.g., transforming a plasmid containing the parent glycosylase into the mutator strain, growing the mutator strain with the plasmid and isolating the mutated plasmid from the mutator strain. The mutated plasmid may be subsequently transformed into the expression organism.

The DNA sequence to be mutagenized may be conveniently present in a genomic or cDNA library prepared from an organism expressing the parent α -amylase. Alternatively, the DNA sequence may be present on a suitable vector such as a plasmid or a bacteriophage, which as such may be incubated with or otherwise exposed to the mutagenising agent. The DNA to be mutagenized may also be present in a host cell either by being integrated in the genome of said cell or by being present on a vector harboured in the cell. Finally, the DNA to be mutagenized may be in isolated form. It will be understood that the DNA sequence to be subjected to random mutagenesis is preferably a cDNA or a genomic DNA sequence.

In some cases it may be convenient to amplify the mutated DNA sequence prior to performing the expression step b) or the screening step c). Such amplification may be performed in accordance with methods known in the art, the presently preferred method being PCR-generated amplification using oligonucleotide primers prepared on the basis of the DNA or amino acid sequence of the parent enzyme.

Subsequent to the incubation with or exposure to the mutagenising agent, the mutated DNA is expressed by culturing a suitable host cell carrying the DNA sequence under conditions allowing expression to take place. The host cell used for this purpose may be one which has been transformed with the mutated DNA sequence, optionally present on a vector, or one which was carried the DNA sequence encoding the parent enzyme during the mutagenesis treatment. Examples of suitable host cells are the following: gram positive bacteria such as *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus lentus*, *Bacillus brevis*, *Bacillus stearothermophilus*, *Bacillus alkalophilus*, *Bacillus amyloliquefaciens*, *Bacillus coagulans*, *Bacillus circulans*, *Bacillus lautus*, *Bacillus megaterium*, *Bacillus thuringiensis*, *Streptomyces lividans* or *Streptomyces murinus*; and gram-negative bacteria such as *E. coli*.

The mutated DNA sequence may further comprise a DNA sequence encoding functions permitting expression of the mutated DNA sequence.

Localized random mutagenesis

The random mutagenesis may be advantageously localized to a part of the parent α -amylase in question. This may, e.g., be advantageous when certain regions of the enzyme have been identified to be of particular importance for a given property of the enzyme, and when modified are expected to result in a variant having improved properties. Such regions may normally be identified when the tertiary structure of the parent enzyme has been elucidated and related to the function of the enzyme.

The localized, or region-specific, random mutagenesis is conveniently performed by use of PCR generated mutagenesis techniques as described above or any other suitable technique known in the art. Alternatively, the DNA sequence encoding the part of the DNA sequence to be modified may be isolated, e.g., by insertion into a suitable vector, and said part may be subsequently subjected to mutagenesis by use of any of the mutagenesis methods discussed above.

10 Alternative methods of providing α -amylase variants

Alternative methods for providing variants of the invention include gene shuffling method known in the art including the methods e.g. described in WO 95/22625 (from Affymax Technologies N.V.) and WO 96/00343 (from Novo Nordisk A/S).

15

Expression of α -amylase variants

According to the invention, a DNA sequence encoding the variant produced by methods described above, or by any alternative methods known in the art, can be expressed, in enzyme form, using an expression vector which typically includes control sequences encoding a promoter, operator, ribosome binding site, translation initiation signal, and, optionally, a repressor gene or various activator genes.

The recombinant expression vector carrying the DNA sequence encoding an α -amylase variant of the invention may be any vector which may conveniently be subjected to recombinant DNA procedures, and the choice of vector will often depend on the host cell into which it is to be introduced. Thus, the vector may be an autonomously replicating vector, i.e. a vector which exists as an extrachromosomal entity, the replication of which is independent of chromosomal replication, e.g. a plasmid, a bacteriophage or an extrachromosomal element, minichromosome or an artificial chromosome. Alternatively, the vector may be one which, when introduced into a host cell, is integrated into the host cell genome and replicated together with the chromosome(s) into which it has been integrated.

In the vector, the DNA sequence should be operably connected

to a suitable promoter sequence. The promoter may be any DNA sequence which shows transcriptional activity in the host cell of choice and may be derived from genes encoding proteins either homologous or heterologous to the host cell. Examples of suitable promoters for directing the transcription of the DNA sequence encoding an α -amylase variant of the invention, especially in a bacterial host, are the promoter of the *lac* operon of *E. coli*, the *Streptomyces coelicolor* agarase gene *dagA* promoters, the promoters of the *Bacillus licheniformis* α -amylase gene (*amyL*), the promoters of the *Bacillus stearothermophilus* maltogenic amylase gene (*amyM*), the promoters of the *Bacillus amyloliquefaciens* α -amylase (*amyQ*), the promoters of the *Bacillus subtilis* *xyIA* and *xyIB* genes etc. For transcription in a fungal host, examples of useful promoters are those derived from the gene encoding *A. oryzae* TAKA amylase, *Rhizomucor miehei* aspartic proteinase, *A. niger* neutral α -amylase, *A. niger* acid stable α -amylase, *A. niger* glucoamylase, *Rhizomucor miehei* lipase, *A. oryzae* alkaline protease, *A. oryzae* triose phosphate isomerase or *A. nidulans* acetamidase.

The expression vector of the invention may also comprise a suitable transcription terminator and, in eukaryotes, polyadenylation sequences operably connected to the DNA sequence encoding the α -amylase variant of the invention. Termination and polyadenylation sequences may suitably be derived from the same sources as the promoter.

The vector may further comprise a DNA sequence enabling the vector to replicate in the host cell in question. Examples of such sequences are the origins of replication of plasmids pUC19, pACYC177, pUB110, pE194, pAMB1 and pIJ702.

The vector may also comprise a selectable marker, e.g. a gene the product of which complements a defect in the host cell, such as the *dal* genes from *B. subtilis* or *B. licheniformis*, or one which confers antibiotic resistance such as ampicillin, kanamycin, chloramphenicol or tetracycline resistance. Furthermore, the vector may comprise *Aspergillus* selection markers such as *amdS*, *argB*, *niaD* and *sC*, a marker giving rise to

hygromycin resistance, or the selection may be accomplished by co-transformation, e.g. as described in WO 91/17243.

While intracellular expression may be advantageous in some respects, e.g. when using certain bacteria as host cells, it is generally preferred that the expression is extracellular. In general, the *Bacillus* α -amylases mentioned herein comprise a preregion permitting secretion of the expressed protease into the culture medium. If desirable, this preregion may be replaced by a different preregion or signal sequence, conveniently accomplished by substitution of the DNA sequences encoding the respective preregions.

The procedures used to ligate the DNA construct of the invention encoding an α -amylase variant, the promoter, terminator and other elements, respectively, and to insert them into suitable vectors containing the information necessary for replication, are well known to persons skilled in the art (cf., for instance, Sambrook et al., *Molecular Cloning: A Laboratory Manual*, 2nd Ed., Cold Spring Harbor, 1989).

The cell of the invention, either comprising a DNA construct or an expression vector of the invention as defined above, is advantageously used as a host cell in the recombinant production of an α -amylase variant of the invention. The cell may be transformed with the DNA construct of the invention encoding the variant, conveniently by integrating the DNA construct (in one or more copies) in the host chromosome. This integration is generally considered to be an advantage as the DNA sequence is more likely to be stably maintained in the cell. Integration of the DNA constructs into the host chromosome may be performed according to conventional methods, e.g. by homologous or heterologous recombination. Alternatively, the cell may be transformed with an expression vector as described above in connection with the different types of host cells.

The cell of the invention may be a cell of a higher organism such as a mammal or an insect, but is preferably a microbial cell, e.g. a bacterial or a fungal (including yeast) cell.

Examples of suitable bacteria are grampositive bacteria such as *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus lentus*,

Bacillus brevis, *Bacillus stearothermophilus*, *Bacillus alkalophilus*, *Bacillus amyloliquefaciens*, *Bacillus coagulans*, *Bacillus circulans*, *Bacillus lautus*, *Bacillus megaterium*, *Bacillus thuringiensis*, or *Streptomyces lividans* or *Streptomyces murinus*, or gramnegative bacteria such as *E.coli*. The transformation of the bacteria may, for instance, be effected by protoplast transformation or by using competent cells in a manner known *per se*.

The yeast organism may favourably be selected from a species of *Saccharomyces* or *Schizosaccharomyces*, e.g. *Saccharomyces cerevisiae*. The filamentous fungus may advantageously belong to a species of *Aspergillus*, e.g. *Aspergillus oryzae* or *Aspergillus niger*. Fungal cells may be transformed by a process involving protoplast formation and transformation of the protoplasts followed by regeneration of the cell wall in a manner known *per se*. A suitable procedure for transformation of *Aspergillus* host cells is described in EP 238 023.

In yet a further aspect, the present invention relates to a method of producing an α -amylase variant of the invention, which method comprises cultivating a host cell as described above under conditions conducive to the production of the variant and recovering the variant from the cells and/or culture medium.

The medium used to cultivate the cells may be any conventional medium suitable for growing the host cell in question and obtaining expression of the α -amylase variant of the invention. Suitable media are available from commercial suppliers or may be prepared according to published recipes (e.g. as described in catalogues of the American Type Culture Collection).

The α -amylase variant secreted from the host cells may conveniently be recovered from the culture medium by well-known procedures, including separating the cells from the medium by centrifugation or filtration, and precipitating proteinaceous components of the medium by means of a salt such as ammonium sulphate, followed by the use of chromatographic procedures such as ion exchange chromatography, affinity chromatography, or the like.

Industrial applications

The α -amylase variants of this invention possesses valuable properties allowing for a variety of industrial applications. In particular, enzyme variants of the invention are applicable as a component in washing, dishwashing and hard-surface cleaning
5 detergent compositions. Numerous variants are particularly useful in the production of sweeteners and ethanol from starch, and/or for textile desizing. Conditions for conventional starch-conversion processes, including starch liquefaction and/or saccharification processes, are described in, e.g., US 3,912,590
10 and in EP patent publications Nos. 252 730 and 63 909.

Production of sweeteners from starch:

A "traditional" process for conversion of starch to fructose syrups normally consists of three consecutive enzymatic
15 processes, viz. a liquefaction process followed by a saccharification process and an isomerization process. During the liquefaction process, starch is degraded to dextrans by an α -amylase (e.g. Termamyl™) at pH values between 5.5 and 6.2 and at temperatures of 95-160°C for a period of approx. 2 hours. In
20 order to ensure an optimal enzyme stability under these conditions, 1 mM of calcium is added (40 ppm free calcium ions).

After the liquefaction process the dextrans are converted into dextrose by addition of a glucoamylase (e.g. AMG™) and a debranching enzyme, such as an isoamylase or a pullulanase (e.g.
25 Promozyme™). Before this step the pH is reduced to a value below 4.5, maintaining the high temperature (above 95°C), and the liquefying α -amylase activity is denatured. The temperature is lowered to 60°C, and glucoamylase and debranching enzyme are added. The saccharification process proceeds for 24-72 hours.

30 After the saccharification process the pH is increased to a value in the range of 6-8, preferably pH 7.5, and the calcium is removed by ion exchange. The dextrose syrup is then converted into high fructose syrup using, e.g., an immobilized glucoisomerase (such as Sweetzyme™).

35 At least 1 enzymatic improvements of this process could be envisaged. Reduction of the calcium dependency of the liquefying α -amylase. Addition of free calcium is required to

ensure adequately high stability of the α -amylase, but free calcium strongly inhibits the activity of the glucoseisomerase and needs to be removed, by means of an expensive unit operation, to an extent which reduces the level of free calcium to below 3-5 ppm. Cost savings could be obtained if such an operation could be avoided and the liquefaction process could be performed without addition of free calcium ions.

To achieve that, a less calcium-dependent Termamyl-like α -amylase which is stable and highly active at low concentrations of free calcium (< 40 ppm) is required. Such a Termamyl-like α -amylase should have a pH optimum at a pH in the range of 4.5-6.5, preferably in the range of 4.5-5.5.

Detergent compositions

As mentioned above, variants of the invention may suitably be incorporated in detergent compositions. Increased thermostability at low calcium concentrations would be very beneficial for amylase performance in detergents, i.e. the alkaline region. Reference is made, for example, to WO 96/23874 and WO 97/07202 for further details concerning relevant ingredients of detergent compositions (such as laundry or dishwashing detergents), appropriate methods of formulating the variants in such detergent compositions, and for examples of relevant types of detergent compositions.

Detergent compositions comprising a variant of the invention may additionally comprise one or more other enzymes, such as a lipase, cutinase, protease, cellulase, peroxidase or laccase, and/or another α -amylase.

α -amylase variants of the invention may be incorporated in detergents at conventionally employed concentrations. It is at present contemplated that a variant of the invention may be incorporated in an amount corresponding to 0.00001-1 mg (calculated as pure, active enzyme protein) of α -amylase per liter of wash/dishwash liquor using conventional dosing levels of detergent.

The invention also relates to a composition comprising

a mixture of one or more variants of the invention derived from (as the parent Termamyl-like α -amylase) the *B. stearothermophilus* α -amylase having the sequence shown in SEQ ID NO: 3 and a Termamyl-like α -amylase derived from the *B. licheniformis* α -amylase having the sequence shown in SEQ ID NO: 4.

Further, the invention also relates to a composition comprising a mixture of one or more variants according the invention derived from (as the parent Termamyl-like α -amylase) the *B. stearothermophilus* α -amylase having the sequence shown in SEQ ID NO: 3 and a hybrid α -amylase comprising a part of the *B. amyloliquefaciens* α -amylase shown in SEQ ID NO: 5 and a part of the *B. licheniformis* α -amylase shown in SEQ ID NO: 4. The latter mentioned hybrid Termamyl-like α -amylase comprises the 445 C-terminal amino acid residues of the *B. licheniformis* α -amylase shown in SEQ ID NO: 4 and the 37 N-terminal amino acid residues of the α -amylase derived from *B. amyloliquefaciens* shown in SEQ ID NO: 5. Said latter mentioned hybrid α -amylase may suitably comprise the following mutations: H156Y+A181T+N190F+A209V+Q264S (using the numbering in SEQ ID NO: 4). In the examples below said hybrid parent Termamyl-like α -amylase, is used in combination with variants of the invention, which variants may be used in compositions of the invention.

In a specific embodiment of the invention the composition comprises a mixture of TVB146 and LE174, e.g., in a ratio of 2:1 to 1:2, such as 1:1.

A α -amylase variant of the invention or a composition of the invention may in an aspect of the invention be used for washing and/or dishwashing; for textile desizing or for starch liquefaction.

MATERIALS AND METHODS

Enzymes:

- BSG alpha-amylase: *B. stearothermophilus* alpha-amylase depicted
5 in SEQ ID NO: 3.
TVB146 alpha-amylase variant: *B. stearothermophilus* alpha-
amylase variant depicted in SEQ ID NO: 3 with the following
mutations: with the deletion in positions I181-G182 + N193F.
LE174 hybrid alpha-amylase variant:
10 LE174 is a hybrid Termamyl-like alpha-amylase being identical
to the Termamyl sequence, i.e., the *Bacillus licheniformis* α -
amylase shown in SEQ ID NO: 4, except that the N-terminal 35
amino acid residues (of the mature protein) has been replaced
by the N-terminal 33 residues of BAN (mature protein), i.e.,
15 the *Bacillus amyloliquefaciens* alpha-amylase shown in SEQ ID
NO: 5, which further have following mutations:
H156Y+A181T+N190F+A209V+Q264S (using the numbering in SEQ ID NO:
4). LE174 was constructed by SOE-PCR (Higuchi et al. 1988,
Nucleic Acids Research 16:7351).

20

Fermentation and purification of α -amylase variants

- A *B. subtilis* strain harbouring the relevant expression
plasmid is streaked on a LB-agar plate with 10 μ g/ml kanamycin
from -80°C stock, and grown overnight at 37°C.
25 The colonies are transferred to 100 ml BPX media supplemented
with 10 μ g/ml kanamycin in a 500 ml shaking flask.

Composition of BPX medium:

- | | |
|--|---------|
| Potato starch | 100 g/l |
| Barley flour | 50 g/l |
| 30 BAN 5000 SKB | 0.1 g/l |
| Sodium caseinate | 10 g/l |
| Soy Bean Meal | 20 g/l |
| Na ₂ HPO ₄ , 12 H ₂ O | 9 g/l |
| Pluronic™ | 0.1 g/l |

35

The culture is shaken at 37°C at 270 rpm for 5 days.

Cells and cell debris are removed from the fermentation broth by centrifugation at 4500 rpm in 20-25 minutes. Afterwards the supernatant is filtered to obtain a completely clear solution.

- 5 The filtrate is concentrated and washed on a UF-filter (10000 cut off membrane) and the buffer is changed to 20mM Acetate pH 5.5. The UF-filtrate is applied on a S-sepharose F.F. and elution is carried out by step elution with 0.2M NaCl in the same buffer. The eluate is dialysed against 10mM Tris, pH 9.0 and applied on a
10 Q-sepharose F.F. and eluted with a linear gradient from 0-0.3M NaCl over 6 column volumes. The fractions which contain the activity (measured by the Phadebas assay) are pooled, pH was adjusted to pH 7.5 and remaining color was removed by a treatment with 0.5% W/vol. active coal in 5 minutes.

15

Activity determination - (KNU)

One Kilo alphah-amylase Unit (1 KNU) is the amount of enzyme which breaks down 5.26 g starch (Merck, Amylum Solubile, Erg. B 6,
20 Batch 9947275) per hour in Novo Nordisk's standard method for determination of alpha-amylase based upon the following condition:

Substrate	soluble starch
25 Calcium content in solvent	0.0043 M
Reaction time	7-20 minutes
Temperature	37°C
pH	5.6

- 30 Detailed description of Novo Nordisk's analytical method (AF 9) is available on request.

BS-amylase Activity Determination - KNU(S)**1. Application Field**

This method is used to determine α -amylase activity in fermentation and recovery samples and formulated and granulated products.

2. Principle

BS-amylase breaks down the substrate (4,6-ethylidene(G₁)-p-nitrophenyl(G₂)- α ,D-maltoheptaoside (written as ethylidene-G₁-PNP) into, among other things, G₂-ENP and G₃-PNP, where G denoted glucose and PNP p-nitrophenol.

G₂-PNP and G₃-PNP are broken down by α -glucosidase, which is added in excess, into glucose and the yellow-coloured p-nitrophenol.

The colour reaction is monitored in situ and the change in absorbance over time calculated as an expression of the speed of the reaction and thus of the activity of the enzyme. See the Boehringer Mannheim 1442 309 guidelines for further details.

2.1 Reaction conditions**Reaction:**

Temperature : 37°C
pH : 7.1
Pre-incubation time: 2 minutes

25 Detection:

Wavelength : 405 nm
Measurement time 3 minutes

3. Definition of Units

Bacillus stearothermophilus α -amylase (BS-amylase) activity is determined relative to a standard of declared activity and stated in Kilo Novo Units (*Stearothermophilus*) or KNU(S)).

4. Specificity and Sensitivity

Limit of determination: approx. 0.4 KNU(s)/g

5. Apparatus

Cobas Fara analyser

Diluted (e.g. Hamilton Microlab 1000)

Analytical balance (e.g. Mettler AE 100)

5 Stirrer plates

6. Reagents/Substrates

A ready-made kit is used in this analysis to determine α -amylase activity. Note that the reagents specified for the substrate and
10 α -glucosidase are not used as described in the Boehringer Mannheim guidelines. However, the designations "buffer", "glass 1", "glass 1a" and Glass 2" are those referred to in those guidelines.

15 6.1. Substrate

4,6-ethylidene(G_1)-p-nitrophenyl(G_1)- α ,D-maltoheptaoside (written as ethylidene- G_1 -PNP) e.g. Boehringer Mannheim 1442 309

6.2 α -glucosidase help reagent

20 α -glucosidase, e.g. Boehringer Mannheim 1442 309

6.3 BRIJ 35 solution

BRIJ 35 (30% W/V Sigma 430 AG-6)

1000 mL

Demineralized water

up to 2,000 mL

25

6.4 Stabiliser

Brij 35 solution

33 mL

$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (Merck 2382)

882 g

Demineralized water

up to 2,000 mL

30

7. Samples and Standards

7.1 Standard curve

35 Example: Preparation of ES-amylase standard curve

The relevant standard is diluted to 0.60 KNU(s)/mL as follows. A calculated quantity of standard is weighed out and added to 200 mL volumetric flask, which is filled to around the 2/3 mark with demineralized water. Stabiliser corresponding to 1% of the volume of the flask is added and the flask is filled to the mark with demineralized water.

A Hamilton Microlab 1000 is used to produce the dilutions shown below. Demineralized water with 1% stabiliser is used as the diluent.

Dilution No.	Enzyme stock solution	1% stabiliser	KNU(s)/mL
1	20µL	580µL	0.02
2	30µL	570µL	0.03
3	40µL	560µL	0.04
4	50µL	550µL	0.05
5	60µL	540µL	0.06

7.2 Level control

A Novo Nordisk A/S BS amylase level control is included in all runs using the Cobas Fara. The control is diluted with 1% stabiliser so that the final dilution is within the range of the standard curve. All weights and dilutions are noted on the worklist

7.3 Sample solutions

Single determination

Fermentation samples (not final samples) from production, all fermentation samples from pilot plants and storage stability samples are weighed out and analyzed once only.

Double determination over 1 run:

Process samples, final fermentation samples from production, samples from GLP studies and R&D samples are weighed out and analyzed twice.

Double determinations over 2 runs:

Finished product samples are weighed out and analyzed twice over two separate runs.

Maximum concentration of samples in powder form: 5%

- 5 Test samples are diluted with demineralized water with 1% stabiliser to approx. 0.037 KNU(S)/mL on the basis of their expected activity. The final dilution is made direct into the sample cup.

8. Procedure

10 8.1 Cobas Menu Program

- The Cobas Menu Program is used to suggest the weight/dilutions of samples and level control to be used.
- The samples are entered into the program with a unique identification code and a worklist is printed out
- 15 ■ The samples and control are weighed out and diluted as stated on the worklist with hand-written weight data is inserted into the BS-amylase analysis logbook
- The results are computered automatically by the Cobas Fara as described in item 9 and printed out along with the standard curve.
- 20 ■ Worklists and results printouts are inserted into the BS-amylase analysis logbook.

8.2 Cobas Fara set-up

- 25 ■ The samples are placed in the sample rack
- The five standards are placed in the calibration rack at position 1 to 5 (strongest standard at position 5), and control placed in the same rack at position 10.
- The substrate is transferred to a 30 mL reagent container and placed in that reagent rack at position 2 (holder 1).
- 30 ■ The α -glucosidase help reagent is transferred to a 50 mL reagent container and placed in the reagent rack at position 2 (holder C)

35 8.3 Cobas Fare analysis

The main principles of the analysis are as follows:

- 20µL sample and 10µL rinse-water are pipetted into the cuvette along with 250µL α-glucosidase help reagent. The cuvette rotates for 10 seconds and the reagents are thrown out into the horizontal cuvettes. 25µL substrate and 20µL rinse-water are pipetted off. After a 1 second wait to ensure that the temperature is 37°C, the cuvette rotates again and the substrate is mixed into the horizontal cuvettes. Absorbance is measured for the first time after 120 seconds and then every 5 seconds. Absorbance is measured a total of 37 times for each sample.

9. Calculations

The activity of the samples is calculated relative to Novo Nordisk A/S standard.

- The standard curve is plotted by the analyzer. The curve is to be gently curved, rising steadily to an absorbance of around 0.25 for standard no. 5.

The activity of the samples in KNU(S)/mL is read off the standard curve by the analyzer.

- The final calculations to allow for the weights/dilutions used employ the following formula:

Activity in KNU(S)/g = $S \times V \times F/W$

S = analysis result read off (KNU(S)/mL

V = volume of volumetric flask used in mL

- F = dilution factor for second dilution

W = weight of enzyme sample in g

9.2 Calculation of mean values

- Results are stated with 3 significant digits. However, for sample activity < 10 KNU(S)/g, only 2 significant digits are given.

The following rules apply on calculation of mean values:

1. Data which deviates more than 2 standard deviations from the mean value is not included in the calculation.
2. Single and double determination over one run:

The mean value is calculated on basis of results lying within the standard curve's activity area.

3. Double determinations over two runs: All values are included in the mean value. Outliers are omitted.

10. Accuracy and Precision

- 5 The coefficient of variation is 2.9% based on retrospective validation of analysis results for a number of finished products and the level control.

Assay for α -Amylase Activity

- 10 α -Amylase activity is determined by a method employing Phadebas® tablets as substrate. Phadebas tablets (Phadebas® Amylase Test, supplied by Pharmacia Diagnostic) contain a cross-linked insoluble blue-coloured starch polymer which has been mixed with bovine serum albumin and a buffer substance and
15 tabletted.

- For every single measurement one tablet is suspended in a tube containing 5 ml 50 mM Britton-Robinson buffer (50 mM acetic acid, 50 mM phosphoric acid, 50 mM boric acid, 0.1 mM CaCl_2 , pH adjusted to the value of interest with NaOH). The test is
20 performed in a water bath at the temperature of interest. The α -amylase to be tested is diluted in x ml of 50 mM Britton-Robinson buffer. 1 ml of this α -amylase solution is added to the 5 ml 50 mM Britton-Robinson buffer. The starch is hydrolysed by the α -amylase giving soluble blue fragments. The absorbance of
25 the resulting blue solution, measured spectrophotometrically at 620 nm, is a function of the α -amylase activity.

- It is important that the measured 620 nm absorbance after 10 or 15 minutes of incubation (testing time) is in the range of 0.2 to 2.0 absorbance units at 620 nm. In this absorbance range
30 there is linearity between activity and absorbance (Lambert-Beer law). The dilution of the enzyme must therefore be adjusted to fit this criterion. Under a specified set of conditions (temp., pH, reaction time, buffer conditions) 1 mg of a given α -amylase will hydrolyse a certain amount of substrate and a blue colour
35 will be produced. The colour intensity is measured at 620 nm. The measured absorbance is directly proportional to the specific

activity (activity/mg of pure α -amylase protein) of the α -amylase in question under the given set of conditions.

EXAMPLES**EXAMPLE 1**Construction of variants of BSG α -amylase (SEQ ID NO: 3)

5 The gene encoding BSG, amyS, is located in plasmid pPL1117. This plasmid contains also the gene conferring resistance towards kanamycin and an origin of replication, both obtained from plasmid pUB110 (Gryczan, T.J. et al (1978) J.Bact 134:318-329).

10 The DNA sequence of the mature part of amyS is shown as SEQ ID NO: 11 and the amino acid sequence of the mature protein is shown as SEQ ID NO: 3

BSG variant TVB145, which contains a deletion of 6 nucleotides corresponding to amino acids I181-G182 in the
15 mature protein, is constructed as follows:

Polymerase Chain Reaction (PCR) is utilized to amplify the part of the amyS gene (from plasmid pPL1117), located between DNA primers BSG1 (SEQ ID NO: 15) and BSGM2 (SEQ ID NO: 16). BSG1 is identical to a part of the amyS gene whereas BSGM2
20 contains the 6 bp nucleotide deletion. A standard PCR reaction is carried out: 94°C for 5 minutes, 25 cycles of (94°C for 45 seconds, 50°C for 45 seconds, 72°C for 90 seconds), 72°C for 7 minutes using the Pwo polymerase under conditions as recommended by the manufacturer, Boehringer Mannheim GmbH.

25 The resulting approximately 550 bp amplified band was used as a megaprimer (Barik, S and Galinski, MS (1991): Biotechniques 10: 489-490) together with primer BSG3 in a second PCR with pPL1117 as template resulting in a DNA fragment of approximately 1080 bp.

30 This DNA fragment is digested with restriction endonucleases Acc65I and SalI and the resulting approximately 550 bp fragment is ligated into plasmid pPL1117 digested with the same enzymes and transformed into the protease- and amylase-deleted *Bacillus subtilis* strain SHA273 (described in
35 WO92/11357 and WO95/10603). Kanamycin resistant and starch degrading transformants were analysed for the presence of the desired mutations (restriction

digest to verify the introduction of a HindIII site in the gene). The DNA sequence between restriction sites Acc65I and SalI was verified by DNA sequencing to ensure the presence of only the desired mutations.

5 BSG variant TVB146 which contains the same 6 nucleotide deletion as TVB145 and an additional substitution of asparagine 193 for a phenylalanine, N193F, was constructed in a similar way as TVB145 utilizing primer BSGM3 (SEQ ID NO: 19) in the first PCR.

10 BSG variant TVB161, containing the deletion of I181-G182, N193F, and L204F, is constructed in a similar way as the two previous variants except that the template for the PCR reactions is plasmid pTVB146 (pPL1117 containing the TVB146-mutations within amyS and the mutagenic oligonucleotide for the first PCR is BSGM3.

15 BSG variant TVB162, containing the deletion of I181-G182, N193F, and E210H, is constructed in a similar way as TVB161 except that the mutagenic oligonucleotide is BSGM4 (SEQ ID NO: 20).

20 BSG variant TVB163, containing the deletion of I181-G182, N193F, and E214Q, is constructed in a similar way as TVB161 except that the mutagenic oligonucleotide is BSGM5 (SEQ ID NO: 21).

25 The above constructed BSG variants were then fermented and purified as described above in the "Material and Methods" section.

EXAMPLE 2

Measurement of the calcium- and pH-dependent stability

30 Normally, the industrial liquefaction process runs using pH 6.0-6.2 as liquefaction pH and an addition of 40 ppm free calcium in order to improve the stability at 95°C-105°C. Some of the herein proposed substitutions have been made in order to improve the stability at

- 35 1. lower pH than pH 6.2 and/or
2. at free calcium levels lower than 40 ppm free calcium.

Two different methods have been used to measure the improvements in stability obtained by the different

substitutions in the α -amylase from *B.stearothermophilus*:

Method 1. One assay which measures the stability at reduced pH, pH 5.0, in the presence of 5 ppm free calcium.

10 μ g of the variant were incubated under the

- 5 following conditions: A 0.1 M acetate solution, pH adjusted to pH 5.0, containing 5ppm calcium and 5% w/w common corn starch (free of calcium). Incubation was made in a water bath at 95°C for 30 minutes.

- Method 2. One assay which measure the stability in the
10 absence of free calcium and where the pH is maintained at pH 6.0. This assay measures the decrease in calcium sensitivity:

10 μ g of the variant were incubated under the following conditions: A 0.1 M acetate solution, pH adjusted to pH 6.0, containing 5% w/w common corn starch (free of
15 calcium). Incubation was made in a water bath at 95°C for 30 minutes.

Stability determination

- All the stability trials 1, 2 have been made using
20 the same set up. The method was:

- The enzyme was incubated under the relevant conditions (1-4). Samples were taken at 0, 5, 10, 15 and 30 minutes and diluted 25 times (same dilution for all taken samples) in assay buffer (0.1M 50mM Britton buffer pH 7.3) and the activity was
25 measured using the Phadebas assay (Pharmacia) under standard conditions pH 7.3, 37°C.

- The activity measured before incubation (0 minutes) was used as reference (100%). The decline in percent was calculated as a function of the incubation time. The table shows the
30 residual activity after 30 minutes of incubation.

Stability method 1. / Low pH stability improvement

MINUTES OF INCUBATION	WT. SEQ. ID. NO:3 AMYLASE (BSG)	SEQ. ID NO: 3 VARIANT WITH DELETION IN POS. I181-G182 (TVB145)	SEQ. ID NO: 3 VARIANT WITH DELETION IN POS. I181-G182 + N193F (TVB146)	SEQ. ID NO: 3 VARIANT WITH DELETION IN POS. I181-G182 + N193F + E214Q (TVB163)
0	100	100	100	100
5	29	71	83	77
10	9	62	77	70
15	3	50	72	67
30	1	33	62	60

5 Stability method 1. / Low pH stability improvement

The temperature described in method 1 has been reduced from 95°C to 70°C since the amylases mentioned for SEQ ID NO: 1 and 2 have a lower thermostability than the one for SEQ ID NO: 3.

MINUTES OF INCUBATION	WT. SEQ. ID. NO: 2 AMYLASE	SEQ. ID NO: 2 VARIANT WITH DELETION IN POS. D183-G184	SEQ. ID NO: 1 AMYLASE	SEQ. ID NO: 1 VARIANT WITH DELETION IN POS. T183-G184
0	100	100	100	100
5	73	92	41	76
10	59	88	19	69
15	48	91	11	62
30	28	92	3	59

Stability method 2. / Low calcium sensitivity

MINUTES OF INCUBATION	WT. SEQ ID NO: 3 AMYLASE (BSG)	SEQ ID NO: 3 VARIANT WITH DELETION IN POS. I181-G182 (TVB145)	SEQ ID NO: 3 VARIANT WITH DELETION IN POS. I181-G182 + N193F (TVB146)	SEQ ID NO: 3 VARIANT WITH DELETION IN POS. I181-G182 + N193F + E214Q (TVE163)
0	100	100	100	100
5	60	82	81	82
10	42	76	80	83
15	31	77	81	79
30	15	67	78	79

Specific activity determination.

- 5 The specific activity was determined using the Phadebas assay (Pharmacia) as activity/mg enzyme. The activity was determined using the α -amylase assay described in the Materials and Methods section herein.

- 10 The specific activity of the parent enzyme and a single and a double mutation was determined to:

BSG: SEQ ID NO:3 (Parent enzyme) 20000 NU/mg

TVB145: SEQ ID NO:3 with the deletion in positions
I181-G182: (Single mutation) 34600 NU/mg

15

TVB146: SEQ ID NO:3 with the deletion in positions
I181-G182 + N193F: (Double mutation)

36600 NU/mg

TVE163: SEQ ID NO:3 with the deletion in positions
20 I181-G182+N193F+E214Q: (Triple mutation) 36300 NU/mg

EXAMPLE 3

Pilot plant jet cook and liquefaction with alpha-amylase

variant TVB146

Pilot plant liquefaction experiments were run in the mini-jet system using a dosage of 50 NU (S)/g DS at pH 5.5 with 5 ppm added Ca^{++} , to compare the performance of formulated BSG alpha-amylase variant TVB146 (SEQ ID NO: 3 with deletion in positions I181-G182 + N193F) with that of parent BSG alpha-amylase (SEQ ID NO: 3). The reaction was monitored by measuring the DE increase (Neocuproine method) as a function of time.

Corn starch slurries were prepared by suspending 11.8 kg Cerestar C*Pharm GL 03406 (89 % starch) in deionized water and making up to 30 kg. The pH was adjusted to 5.5 at ambient temperature, after the addition of 0.55 g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$.

The following enzymes were used:

TVB146	108 KNU(S)/g, 146 KNU(SM9)/g
BSG amylase	101 KNU(S)/g, 98 KNU(SM9)/g

An amount of enzyme corresponding to 50 NU (SM9)/g DS was added, and the conductivity adjusted to 300mS using NaCl. The standard conditions were as follows:

Substrate concentration	35 % w/w (initial) 31.6-31.9 % w/w (final)
Temperature	105°C, 5 min (Primary liquefaction) 95°C, 90 min (Secondary liquefaction)
pH (initial)	5.5

After jetting, the liquefied starch was collected and transported in sealed thermos-flasks from the pilot plant to the laboratory, where secondary liquefaction was continued at 95 °C.

10 ml samples were taken at 15 minute intervals from 15-90 minutes. 2 drops of 1 N HCl were added to inactivate the enzyme. From these samples, 0.3-0.1 g (according to the expected DE) were weighed out and diluted to 100 ml. Reducing sugars were then determined according to the Neocuproine method (Determination of reducing sugar with improved precision).

Dygert, Li, Florida and Thomas (1965). Anal. Biochem 13, 368) and DE values determined. The development of DE as a function of time is given in the following table:

Time (min.)	TVB146	BSG
	DE (neocuproine)	
15	2.80	2.32
30	4.88	3.56
45	6.58	4.98
60	8.17	6.00
75	9.91	7.40
90	11.23	8.03

As can be seen the alpha-amylase variant TVB146 performed significantly better under industrially relevant application conditions at low levels of calcium than the parent BSG alpha-amylase.

EXAMPLE 4

Jet Cook and Liquefaction with a combination of alpha-amylase variants (TVB146 and LE174)

Jet cook and liquefaction using a combination of the alpha-amylase variants, TVB146 and LE174 (ratio 1:1) were carried out at the following conditions:

Substrate A.E. Staley food grade powdered corn starch (100lbs)

D.S. 35% using DI water

Free Ca²⁺ 2.7ppm at pH 5.3 (none added, from the starch only)

Initial pH 5.3

Dose AF9 units (AF9 is available on request) for each enzyme variant was 28 NU/g starch db for a total dose of 56 NU/g

Temperature in primary liquefaction 105°C

Hold time in primary liquefaction 5 minutes

Temperature in secondary liquefaction 95°C

At 15 minutes into secondary liquefaction 1.5 gms of hydrolyzate was added to a tared one liter volumetric containing 500cc of DI water and 1 ml of one normal HCl and the exact wt. added was recorded. This was repeated at 15 minute intervals out to 90 minutes with an additional point at 127

minutes. These were diluted to one liter and determined for dextrose equivalence via Neocuproine method as discribed by Dygert, Li, Florida and Thomas. Determination of reducing sugar with improved precision (1965). Anal. Biochem 13, 368.

5

The results were as follows:

	Time	DE
	15	3.2
	30	4.8
10	45	6.3
	60	7.8
	75	9.4
	90	10.4
	127	13.1

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CLAIMS

1. A variant of a parent Termamyl-like α -amylase with α -amylase activity comprising mutations in two, three, four, five or six of the following regions/positions or in corresponding positions in other parent Termamyl-like α -amylases:

(relative to SEQ ID NO: 1):

1: R181*, G182*, T183*, G184*

2: N195A, R, D, C, E, Q, G, H, I, L, K, M, F, P, S, T, W, Y, V;

10 3: V206A, R, D, N, C, E, Q, G, H, I, L, K, M, F, P, S, T, W, Y, V;

4: E212A, R, D, N, C, Q, G, H, I, L, K, M, F, P, S, T, W, Y, V;

5: E216A, R, D, N, C, Q, G, H, I, L, K, M, F, P, S, T, W, Y, V;

6: K269A, R, D, N, C, E, Q, G, H, I, L, M, F, P, S, T, W, Y, V;

(relative to SEQ ID NO: 2):

15 1: R181*, G182*, D183*, G184*

2: N195A, R, D, C, E, Q, G, H, I, L, K, M, F, P, S, T, W, Y, V;

3: V206A, R, D, N, C, E, Q, G, H, I, L, K, M, F, P, S, T, W, Y, V;

4: E212A, R, D, N, C, Q, G, H, I, L, K, M, F, P, S, T, W, Y, V;

5: E216A, R, D, N, C, Q, G, H, I, L, K, M, F, P, S, T, W, Y, V;

20 6: K269A, R, D, N, C, E, Q, G, H, I, L, M, F, P, S, T, W, Y, V;

(Relative to SEQ ID NO: 3):

1: R179*, G180, I181*, G182*

2: N193A, R, D, C, E, Q, G, H, I, L, K, M, F, P, S, T, W, Y, V;

3: L204A, R, D, N, C, E, Q, G, H, I, K, M, F, P, S, T, W, Y, V;

25 4: E210A, R, D, N, C, Q, G, H, I, L, K, M, F, P, S, T, W, Y, V;

5: E214A, R, D, N, C, Q, G, H, I, L, K, M, F, P, S, T, W, Y, V;

6: S267A, R, D, N, C, E, Q, G, H, I, L, K, M, F, P, T, W, Y, V

Relative to SEQ ID NO: 4):

1: N190A, R, D, C, E, Q, G, H, I, L, K, M, F, P, S, T, W, Y, V;

30 2: I201A, R, D, N, C, E, Q, G, H, L, K, M, F, P, S, T, W, Y, V;

3: D207A, R, N, C, E, Q, G, H, I, L, K, M, F, P, S, T, W, Y, V;

4: E211A, R, D, N, C, Q, G, H, I, L, K, M, F, P, S, T, W, Y, V;

5: Q264A, R, D, N, C, E, Q, G, H, I, L, K, M, F, P, S, T, W, Y, V;

(relative to SEQ ID NO: 5):

35 1: R176*, G177*, E178, G179*

2: N190A, R, D, C, E, Q, G, H, I, L, K, M, F, P, S, T, W, Y, V;

3: V201A, R, D, N, C, E, Q, G, H, I, L, K, M, F, P, S, T, W, Y, V;

4: D207A,R,N,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
5: E211A,R,D,N,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
6: Q264A,R,D,N,C,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
(relative to SEQ ID NO: 6):

- 5 1: R181*,G182*,H183*,G184*
2: N195A,R,D,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
3: I206A,R,D,N,C,E,Q,G,H,L,K,M,F,P,S,T,W,Y,V;
4: E212A,R,D,N,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
5: E216A,R,D,N,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
10 6: K269A,R,D,N,C,E,Q,G,H,I,L,M,F,P,S,T,W,Y,V;

2. The variant according to claim 1, comprising the following mutations: N190F/Q264S in SEQ ID NO: 4 or in corresponding positions in another parent α -amylase.

15

3. The variant according to claim 1, comprising the following mutations: I181*/G182*/N193F in SEQ ID NO: 3 or in corresponding positions in another parent Termamyl like α -amylase.

- 20 4. The variant according to claim 3, further comprising a substitution in position E214Q in SEQ ID NO: 3 or in a corresponding position in another parent Termamyl like α -amylase.

- 25 5. The variant according to any of claims 1 to 4, wherein the parent α -amylase is a hybrid α -amylase of SEQ ID NO: 4 and SEQ ID NO: 5.

6. The variant according to claim 5, wherein the parent hybrid
30 α -amylase is a hybrid alpha-amylase comprising the 445 C-terminal amino acid residues of the *B. licheniformis* α -amylase shown in SEQ ID NO: 4 and the 37 N-terminal amino acid residues of the α -amylase derived from *B. amyloliquefaciens* shown in SEQ ID NO: 5.

35

7. The variant according to claim 6, wherein the parent hybrid

Termamyl-like α -amylase further has the following mutations:
H156Y+A181T+N190F+A209V+Q264S (using the numbering in SEQ ID NO:
4).

5 8. The variant according to claim 1, exhibiting increased
stability at acidic pH and/or low Ca^{2+} concentration:

9. A DNA construct comprising a DNA sequence encoding an α -
amylase variant according to any one of claims 1 to 8.

10

10. A recombinant expression vector which carries a DNA con-
struct according to claim 9.

15

11. A cell which is transformed with a DNA construct according
to claim 9 or a vector according to claim 10.

12. A cell according to claim 11, which is a microorganism.

20

13. A cell according to claim 12, which is a bacterium or a
fungus.

25

14. The cell according to claim 13, which is a grampositive
bacterium such as *Bacillus subtilis*, *Bacillus licheniformis*,
Bacillus lentus, *Bacillus brevis*, *Bacillus stearothermophilus*,
Bacillus alkalophilus, *Bacillus amyloliquefaciens*, *Bacillus*
coagulans, *Bacillus circulans*, *Bacillus lautus* or *Bacillus thu-*
ringiensis.

30

15. A detergent additive comprising an α -amylase variant accor-
ding to any one of claims 1 to 8, optionally in the form of a
non-dusting granulate, stabilised liquid or protected enzyme.

35

16. A detergent additive according to claim 15 which contains
0.02-200 mg of enzyme protein/g of the additive.

17. A detergent additive according to claims 15 or 16, which
additionally comprises another enzyme such as a protease, a

lipase, a peroxidase, another amylolytic enzyme and/or a cellulase.

18. A detergent composition comprising an α -amylase variant according to any of claims 1 to 8.

19. The detergent composition according to claim 18 which additionally comprises another enzyme such as a protease, a lipase, a peroxidase, another amylolytic enzyme and/or a cellulase.

20. A manual or automatic dishwashing detergent composition comprising an α -amylase variant according to any one of claims 1 to 8.

21. A dishwashing detergent composition according to claim 20 which additionally comprises another enzyme such as a protease, a lipase, a peroxidase, another amylolytic enzyme and/or a cellulase.

22. A manual or automatic laundry washing composition comprising an α -amylase variant according to any one of claims 1 to 8.

23. A laundry washing composition according to claim 22, which additionally comprises another enzyme such as a protease, a lipase, a peroxidase, an amylolytic enzyme and/or a cellulase.

24. A composition comprising:

(i) a mixture of the α -amylase from *B. licheniformis* having the sequence shown in SEQ ID NO: 4 with one or more variants according to any of claims 1 to 8 derived from (as the parent Termamyl-like α -amylase) the *B. stearotherophilus* α -amylase having the sequence shown in SEQ ID NO: 3; or

(ii) a mixture of the α -amylase from *B. stearotherophilus* having the sequence shown in SEQ ID NO: 3 with one or more variants according to any of claims 1 to 8 derived from one or more other parent Termamyl-like α -amylases; or

- (iii) a mixture of one or more variants according any of claim 1 to 8 derived from (as the parent Termamyl-like α -amylase) the *B. stearothermophilus* α -amylase having the sequence shown in SEQ ID NO: 3 with one or more variants according to the invention
3 derived from one or more other parent Termamyl-like α -amylases.

25. A composition comprising:

- a mixture of one or more variants according any of claims 1 to 8 derived from (as the parent Termamyl-like α -amylase) the *B. stearothermophilus* α -amylase having the sequence shown in SEQ ID
10 NO: 3 and a Termamyl-like alpha-amylase derived from the *B. licheniformis* α -amylase having the sequence shown in SEQ ID NO: 4.

15 26. The composition comprising:

- a mixture of one or more variants according any of claims 1 to 8 derived from (as the parent Termamyl-like α -amylase) the *B. stearothermophilus* α -amylase having the sequence shown in SEQ ID NO: 3 and a hybrid alpha-amylase comprising a part of the *B. amyloliquefaciens* α -amylase shown in SEQ ID NO: 5 and a part of
20 the *B. licheniformis* α -amylase shown in SEQ ID NO: 4.

27. The composition according to claim 26, wherein the hybrid α -amylase is a hybrid alpha-amylase comprising the 445 C-terminal
25 amino acid residues of the *B. licheniformis* α -amylase shown in SEQ ID NO: 4 and the 37 N-terminal amino acid residues of the α -amylase derived from *B. amyloliquefaciens* shown in SEQ ID NO: 5.

28. The composition according to claim 27, wherein the hybrid α -amylase further has the following mutations:
30 H156Y+A181T+N190F+A209V+Q264S (using the numbering in SEQ ID NO: 4).

29. The composition according to claims 26, comprising a mixture

of TVB146 and LE174.

30. Use of an α -amylase variant according to any of claims 1 to
8 or a composition according to any of claims 24 to 29 for
5 washing and/or dishwashing.

31. Use of an α -amylase variant according to any of claims 1 to
8 or a composition according to any of claims 24 to 29 for
textile desizing.

10

32. Use of an α -amylase variant according to any of claims 1 to
8 or a composition according to any of claims 24 to 29 for
starch liquefaction.

15 33. A method for generating a variant of a parent Termamyl-like
 α -amylase, which variant exhibits increased stability at low pH
and at low calcium concentration relative to the parent, the
method comprising:

(a) subjecting a DNA sequence encoding the parent Termamyl-like
20 α -amylase to random mutagenesis,

(b) expressing the mutated DNA sequence obtained in step (a) in
a host cell, and

(c) screening for host cells expressing a mutated α -amylase
which has increased stability at low pH and low calcium
25 concentration relative to the parent α -amylase.

1	HHNGTNGTMM	QYFEWHL PND	GNHWNRLRDD	ASNLNRGKIT	AIWIPPAWK	50
2	..NGTNGTMM	QYFEWYLPND	GNHWNRLRSD	ASNLKDRGIS	AVWIPPAWK	
3	HHNGTNGTMM	QYFEWYLPND	GNHWNRLRDD	AANLKSIGIT	AVWIPPAWK	
4	...VNGTMM	QYFEWYLPND	GOHWKRLQND	AHLSDICIT	AVWIPPAWK	
5	..ANLNGTMM	QYFEWYLPND	GOHWKRLQND	SAYLAHIGIT	AVWIPPAWK	
6	..AAPFNGTMM	QYFEWYLPND	GTWTKVANE	ANLSSIGIT	ALWLPFAWK	
51						100
1	TSQNDVG YGA	YDLYDLGEFN	QKGTVRTKYG	TRSQLES A IH	ALKNNGVQVY	
2	ASQNDVG YGA	YDLYDLGEFN	QKGTVRTKYG	TRNQLQA V N	ALKNGIQVY	
3	TSQNDVG YGA	YDLYDLGEFN	QKGTVRTKYG	TRNQLQA V T	SLKNGIQVY	
4	LSQSDNG YGP	YDLYDLGEFQ	QKGTVRTKYG	TKSELQDA IG	SLHNRNVQVY	
5	TSQADVG YGA	YDLYDLGEFH	QKGTVRTKYG	TKGELQSA IK	SLHSDRIN VY	
6	TSRSDVG YGV	YDLYDLGEFN	QKGTVRTKYG	TKAQYLA IQ	AAHAAQM VY	
101						150
1	GDVVMNHKGG	ADATENVLAV	EVPNNRNQOE	ISGDYTTIEAW	TKDFEPGRGN	
2	GDVVMNHKGG	ADATENVRVAV	EVPNNRNQOE	VSGEYTTIEAW	TKDFEPGRGN	
3	GDVVMNHKGG	ADGTEIVNAV	EVPNSNRNQE	TSGEYATIEAW	TKDFEPGRGN	
4	GDVVLNHKAG	ADATEDVTAV	EVPNANRNQE	TSEYQIKAW	TDFEPGRGN	
5	GDVVLNHKGG	ADATEDVTAV	EVPNADRNRY	ISGEHLIKAW	THEFPGRGS	
6	ADVVFHKG	ADGTEWVDVAV	EVPNSDRNQE	ISGTQIQAW	TKDFEPGRGN	
151						200
1	TSYDFKRWY	HFDGVDWDQS	RQFNRIYKF	RGDGKAWDE	VDSNGNYDY	
2	THSNFKRWY	HFDGVDWDQS	RKLNNRIYKF	RGDGKAWDE	VDTEGNYDY	
3	NHSSFKRWY	HFDGTDWDQS	ROLQNKIYKF	RGTGKAWDE	VDTEGNYDY	
4	TSYDFKRWY	HFDGADWDES	RKI.SRIYKF	RGEKAWDE	VSSNGNYDY	
5	TSYDFKRWY	HFDGTDWDES	RKL.NRIYKF	.QCKAWDE	VSNNGNYDY	
6	TSYDFKRWY	HFDGVDWDES	RKL.SRIYKF	RGIGKAWDE	VDTEGNYDY	

Fig. 1

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5	1	LMYADVDMDH	PEVVNELRRW	GEWYNTNLNL	DGFRIDAVKH	IKYSTFRDNL	250
	2	LMYADVDMDH	PEVVNELRNW	GVWYNTNLGL	DGFRIDAVKH	IKYSTFRDNL	
	3	LMYADVDMDH	PEVIHELNRW	GVWYNTNLNL	DGFRIDAVKH	IKYSTFRDNL	
10	4	LMYADVDDYDH	PDVVAETKKW	GIWYANELSL	DGFRIDAVKH	IKESFLRDWV	
	5	LMYADIDVDH	PDVAAEIKRW	GTWYANELQL	DGFRIDAVKH	IKESFLRDWV	
	6	LMYADLDMDH	PEVVTLEKNW	GWVYNTNITN	DGFRIDAVKH	IKESFPDNL	
	251	THVRNATGKE	MFVAEEFWKN	DIGALENYLN	KTNNHHSVFD	VPLHYNLYNA	300
15	1	IHVRSATCKN	MEVAEEFWKN	DIGALENYLN	KTNNHHSVFD	VPLHYNLYNA	
	2	THVRNTGKCP	MFVAEEFWKN	DIGALENYLN	KTNNHHSVFD	VPLHYNLYNA	
	3	QAVRQATCKE	MFTVAEYQON	NAGKLENYLN	KTNNHHSVFD	VPLHYNLYNA	
	4	NHVRKGTGKE	MFTVAEYQON	DIGALENYLN	KTNNHHSVFD	VPLHYNLYNA	
	5	SYVRSQTGKE	LFTVGEYWSY	DINKLHNYIT	KTNNHHSVFD	VPLHYNLYNA	
20	6						
	301	SNSGGNYDMA	KLINGTVVQK	HPMHAVTFVD	NHDSQGEAL	ESFVQEWFKP	350
	1	SKSGGNYDMR	QIFNGTVVQR	HPMHAVTFVD	NHDSQGEAL	ESFVQEWFKP	
	2	SKSGGNYDMR	NILNGSVVQK	HPMHAVTFVD	NHDSQGEAL	ESFVQEWFKP	
	3	SSQGGGYDMR	RLLDGTVVSR	HPKAVTFVE	NHDSQGEAL	ESTVQWTFKP	
25	4	STQGGGYDMR	KLINGTVVSK	HPKAVTFVE	NHDSQGEAL	ESTVQWTFKP	
	5	SKSGGAEDMR	TLMTNTLMKD	QETLAVTFVD	NHDSQGEAL	QSWVDPWFKP	
	6						
	351	LAYALILTRE	QGYPSVFYGD	YGIPTHS..	.VPAMKAKID	PILEARQNYA	400
30	1	LAYALILTRE	QGYPSVFYGD	YGIPTHG..	.VPAMKSKID	PILEARQNYA	
	2	LAYALILTRE	QGYPSVFYGD	YGIPTHG..	.VPAMKSKID	PILEARQNYA	
	3	LAYALILTRE	SGYPQVFYGD	MYGKTGSPK	EIPSLKDNIE	PILKARQNYA	
	4	LAYALILTRE	SGYPQVFYGD	MYGKTGSDQR	EIPALKHKIE	PILKARQNYA	
35	5	LAYALILTRE	EGYPCVFYGD	YGIPOVN..	.IPSLKSKID	PILLIARQNYA	
	6						

Figure 1 (continued)

Fig. 1

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401	1	YGTOHDYFDH	HNLIQWTREG	NTTHPNSGLA	TIMSDGPGGE	KMYVVCQNK	450
	2	YGRQN.....	
	3	YGTOHDYFDH	HDIIGWTREG	NSSHPSNSGLA	TIMSDGPGGN	KMYVVGKNA	
5	4	YGPQHDYIDH	PDVIGWTREG	DSSAAKSGLA	ALITDGPFGS	KMYVAGLKNA	
	5	YGAQHDYFDH	HDIIGWTREG	DSSVANSGLA	ALITDGPFGA	KMYVVRQNA	
	6	YGTQHDYLDH	SDIIGWTREG	GTEKPGSGLA	ALITDGPFGS	KMYVVRQHA	
10	451	GQVWHIDITGN	KPGTVTINAD	GWANFSVNGG	SVSIWVKR..	500
	1	
	2	GQVWRDITGN	RTGTVTINAD	GWGNFSVNGG	SVSVWVRQ..	
	3	
	4	GETWYDITGN	RSDTVKIGSD	GWGEFHVNDG	SVSIYVQR..	
15	5	GETWHDITGN	RSEPVVINSE	GWGEFHVNGG	SVSIYVQR..	
	6	GKVEYDITGN	RSDTVTINSD	GWGEFVNGG	SVSVWVPRKT	TVSTIARPIT	
501	519	
	1	
20	2	
	3	
	4	
	5	
25	6	TRPWTGEFVR	WTEPRLVAW	

Figure 1 (continued)

Fig. 1

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:

- 5 (A) NAME: NOVO NORDISK A/S
 (B) STREET: Novo Allé
 (C) CITY: DK-2860 Bagsvaerd
 (E) COUNTRY: Denmark
 (F) POSTAL CODE (ZIP): DK-2860
 10 (G) TELEPHONE: +45 44 44 88 88
 (H) TELEFAX: +45 44 49 32 56
 (ii) TITLE OF INVENTION: AMYLASE VARIANTS
 (iii) NUMBER OF SEQUENCES: 21
 (iv) COMPUTER READABLE FORM:
 15 (A) MEDIUM TYPE: Floppy disk
 (B) COMPUTER: IBM PC compatible
 (C) OPERATING SYSTEM: PC-DOS/MS-DOS
 (D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPG)

20 (2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 485 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: single
 25 (D) TOPOLOGY: linear
 (ii) MOLECULE TYPE: peptide
 (iii) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

30 His His Asn Gly Thr Asn Gly Thr Met Met Gln Tyr Phe Glu Trp Tyr
 1 5 10 15
 Leu Pro Asn Asp Gly Asn His Trp Asn Arg Leu Arg Asp Asp Ala Ala
 20 25 30
 35 Asn Leu Lys Ser Lys Gly Ile Thr Ala Val Trp Ile Pro Pro Ala Trp
 35 40 45
 Lys Gly Thr Ser Gln Asn Asp Val Gly Tyr Gly Ala Tyr Asp Leu Tyr
 50 55 60
 40 Asp Leu Gly Glu Phe Asn Gln Lys Gly Thr Val Arg Thr Lys Tyr Gly
 65 70 75 80
 Thr Arg Asn Gln Leu Gln Ala Ala Val Thr Ser Leu Lys Asn Asn Gly
 45 85 90 95
 Ile Gln Val Tyr Gly Asp Val Val Met Asn His Lys Gly Gly Ala Asp
 100 105 110
 50 Gly Thr Glu Ile Val Asn Ala Val Glu Val Asn Arg Ser Asn Arg Asn
 115 120 125
 Gln Glu Thr Ser Gly Glu Tyr Ala Ile Glu Ala Trp Thr Lys Phe Asp
 130 135 140

Phe Pro Gly Arg Gly Asn Asn His Ser Ser Phe Lys Trp Arg Trp Tyr
 145 150 155 160
 5 His Phe Asp Gly Thr Asp Trp Asp Gln Ser Arg Gln Leu Gln Asn Lys
 165 170 175
 Ile Tyr Lys Phe Arg Gly Thr Gly Lys Ala Trp Asp Trp Glu Val Asp
 180 185 190
 10 Thr Glu Asn Gly Asn Tyr Asp Tyr Leu Met Tyr Ala Asp Val Asp Met
 195 200 205
 Asp His Pro Glu Val Ile His Glu Leu Arg Asn Trp Gly Val Trp Tyr
 210 215 220
 15 Thr Asn Thr Leu Asn Leu Asp Gly Phe Arg Ile Asp Ala Val Lys His
 225 230 235 240
 20 Ile Lys Tyr Ser Phe Thr Arg Asp Trp Leu Thr His Val Arg Asn Thr
 245 250 255
 Tor Gly Lys Pro Met Phe Ala Val Ala Glu Phe Trp Lys Asn Asp Leu
 260 265 270
 25 Gly Ala Ile Glu Asn Tyr Leu Asn Lys Thr Ser Trp Asn His Ser Val
 275 280 285
 Phe Asp Val Pro Leu His Tyr Asn Leu Tyr Asn Ala Ser Asn Ser Gly
 290 295 300
 30 Gly Tyr Tyr Asp Met Arg Asn Ile Leu Asn Gly Ser Val Val Gln Lys
 305 310 315 320
 35 His Pro Thr His Ala Val Thr Phe Val Asp Asn His Asp Ser Gln Pro
 325 330 335
 Gly Glu Ala Leu Glu Ser Phe Val Gln Gln Trp Phe Lys Pro Leu Ala
 340 345 350
 40 Tyr Ala Leu Val Leu Thr Arg Glu Gln Gly Tyr Pro Ser Val Phe Tyr
 355 360 365
 Gly Asp Tyr Tyr Gly Ile Pro Thr His Gly Val Pro Ala Met Lys Ser
 370 375 380
 45 Lys Ile Asp Pro Leu Leu Gln Ala Arg Gln Thr Phe Ala Tyr Gly Thr
 385 390 395 400
 50 Gln His Asp Tyr Phe Asp His His Asp Ile Ile Gly Trp Thr Arg Glu
 405 410 415
 Gly Asn Ser Ser His Pro Asn Ser Gly Leu Ala Thr Ile Met Ser Asp
 420 425 430
 55 Gly Pro Gly Gly Asn Lys Trp Met Tyr Val Gly Lys Asn Lys Ala Gly

435 440 445
 Gln Val Trp Arg Asp Ile Thr Gly Asn Arg Thr Gly Thr Val Thr Ile
 450 455 460
 5 Asn Ala Asp Gly Trp Gly Asn Phe Ser Val Asn Gly Gly Ser Val Ser
 465 470 475 480
 10 Val Trp Val Lys Gln
 485
 (2) INFORMATION FOR SEQ ID NO: 2:
 (i) SEQUENCE CHARACTERISTICS:
 15 (A) LENGTH: 485 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear
 (ii) MOLECULE TYPE: peptide
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:
 20 His His Asn Gly Thr Asn Gly Thr Met Met Gln Tyr Phe Glu Trp His
 1 5 10 15
 25 Leu Pro Asn Asp Gly Asn His Trp Asn Arg Leu Arg Asp Asp Ala Ser
 20 25 30
 Asn Leu Arg Asn Arg Gly Ile Thr Ala Ile Trp Ile Pro Pro Ala Trp
 35 40 45
 30 Lys Gly Thr Ser Gln Asn Asp Val Gly Tyr Gly Ala Tyr Asp Leu Tyr
 50 55 60
 Asp Leu Gly Glu Phe Asn Gln Lys Gly Thr Val Arg Thr Lys Tyr Gly
 65 70 75 80
 35 Thr Arg Ser Gln Leu Glu Ser Ala Ile His Ala Leu Lys Asn Asn Gly
 85 90 95
 Val Gln Val Tyr Gly Asp Val Val Met Asn His Lys Gly Gly Ala Asp
 100 105 110
 40 Ala Thr Glu Asn Val Leu Ala Val Glu Val Asn Pro Asn Asn Arg Asn
 115 120 125
 45 Gln Glu Ile Ser Gly Asp Tyr Thr Ile Glu Ala Trp Thr Lys Phe Asp
 130 135 140
 Phe Pro Gly Arg Gly Asn Thr Tyr Ser Asp Phe Lys Trp Arg Trp Tyr
 145 150 155 160
 50 His Phe Asp Gly Val Asp Trp Asp Gln Ser Arg Gln Phe Gln Asn Arg
 165 170 175
 Ile Tyr Lys Phe Arg Gly Asp Gly Lys Ala Trp Asp Trp Glu Val Asp
 180 185 190

Ser Glu Asn Gly Asn Tyr Asp Tyr Leu Met Tyr Ala Asp Val Asp Met
 195 200 205
 5 Asp His Pro Glu Val Val Asn Glu Leu Arg Arg Trp Gly Glu Trp Tyr
 210 215 220
 Thr Asn Thr Leu Asn Leu Asp Gly Phe Arg Ile Asp Ala Val Lys His
 225 230 235 240
 10 Ile Lys Tyr Ser Phe Thr Arg Asp Trp Leu Thr His Val Arg Asn Ala
 245 255
 Thr Gly Lys Glu Met Phe Ala Val Ala Glu Phe Trp Lys Asn Asp Leu
 260 265 270
 15 Gly Ala Leu Glu Asn Tyr Leu Asn Lys Thr Asn Trp Asn His Ser Val
 275 280 285
 Phe Asp Val Pro Leu His Tyr Asn Leu Tyr Asn Ala Ser Asn Ser Gly
 290 295 300
 20 Gly Asn Tyr Asp Met Ala Lys Leu Leu Asn Gly Thr Val Val Gln Lys
 305 310 315 320
 25 His Pro Met His Ala Val Thr Phe Val Asp Asn His Asp Ser Gln Pro
 325 330 335
 Gly Gln Ser Leu Gln Ser Phe Val Gln Glu Trp Phe Lys Pro Leu Ala
 340 345 350
 30 Tyr Ala Leu Ile Leu Thr Arg Glu Gln Gly Tyr Pro Ser Val Phe Tyr
 355 360 365
 35 Gly Asp Tyr Tyr Gly Ile Pro Thr His Ser Val Pro Ala Met Lys Ala
 370 375 380
 Lys Ile Asp Pro Ile Leu Glu Ala Arg Gln Asn Phe Ala Tyr Gly Thr
 385 390 395 400
 40 Gln His Asp Tyr Phe Asp His His Asn Ile Ile Gly Trp Thr Arg Glu
 405 410 415
 Gly Asn Thr Thr His Pro Asn Ser Gly Leu Ala Thr Ile Met Ser Asp
 420 425 430
 45 Gly Pro Gly Gly Glu Lys Trp Met Tyr Val Gly Gln Asn Lys Ala Gly
 435 440 445
 50 Gln Val Trp His Asp Ile Thr Gly Asn Lys Pro Gly Thr Val Thr Ile
 450 455 460
 Asn Ala Asp Gly Trp Ala Asn Phe Ser Val Asn Gly Gly Ser Val Ser
 465 470 475 480
 55

Ile Trp Val Lys Arg
485

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 514 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

Ala Ala Pro Phe Asn Gly Thr Met Met Gln Tyr Phe Glu Trp Tyr Leu
1 5 10 15

Pro Asp Asp Gly Thr Leu Trp Thr Lys Val Ala Asn Glu Ala Asn Asn
20 25 30

Leu Ser Ser Leu Gly Ile Thr Ala Leu Trp Leu Pro Pro Ala Tyr Lys
35 40 45

Gly Thr Ser Arg Ser Asp Val Gly Tyr Gly Val Tyr Asp Leu Tyr Asp
50 55 60

Leu Gly Glu Phe Asn Gln Lys Gly Ala Val Arg Thr Lys Tyr Gly Thr
65 70 75 80

Lys Ala Gln Tyr Leu Gln Ala Ile Gln Ala Ala His Ala Ala Gly Met
85 90 95

Gln Val Tyr Ala Asp Val Val Phe Asp His Lys Gly Gly Ala Asp Gly
100 105 110

Thr Glu Trp Val Asp Ala Val Glu Val Asn Pro Ser Asp Arg Asn Gln
115 120 125

Glu Ile Ser Gly Thr Tyr Gln Ile Gln Ala Trp Thr Lys Phe Asp Phe
130 135 140

Pro Gly Arg Gly Asn Thr Tyr Ser Ser Phe Lys Trp Arg Trp Tyr His
145 150 155 160

Phe Asp Gly Val Asp Trp Asp Glu Ser Arg Lys Leu Ser Arg Ile Tyr
165 170 175

Lys Phe Arg Gly Ile Gly Lys Ala Trp Asp Trp Glu Val Asp Thr Glu
180 185 190

Asn Gly Asn Tyr Asp Tyr Leu Met Tyr Ala Asp Leu Asp Met Asp His
195 200 205

Pro Glu Val Val Thr Glu Leu Lys Ser Trp Gly Lys Trp Tyr Val Asn
210 215 220

Thr Thr Asn Ile Asp Gly Phe Arg Leu Asp Ala Val Lys His Ile Lys

	225		230		235		240
	Phe Ser Phe Phe Pro Asp Trp Leu Ser Asp Val Arg Ser Gln Thr Gly						
		245			250		255
5	Lys Pro Leu Phe Thr Val Gly Glu Tyr Trp Ser Tyr Asp Ile Asn Lys						
		260			265		270
10	Leu His Asn Tyr Ile Met Lys Thr Asn Gly Thr Met Ser Leu Phe Asp						
		275			280		285
	Ala Pro Leu His Asn Lys Phe Tyr Thr Ala Ser Lys Ser Gly Gly Thr						
		290			295		300
15	Phe Asp Met Arg Thr Leu Met Thr Asn Thr Leu Met Lys Asp Gln Pro						
		305			310		315
	Thr Leu Ala Val Thr Phe Val Asp Asn His Asp Thr Glu Pro Gly Gln						
		325			330		335
20	Ala Leu Gln Ser Trp Val Asp Pro Trp Phe Lys Pro Leu Ala Tyr Ala						
		340			345		350
25	Phe Ile Leu Thr Arg Gln Glu Gly Tyr Pro Cys Val Phe Tyr Gly Asp						
		355			360		365
	Tyr Tyr Gly Ile Pro Gln Tyr Asn Ile Pro Ser Leu Lys Ser Lys Ile						
		370			375		380
30	Asp Pro Leu Leu Ile Ala Arg Arg Asp Tyr Ala Tyr Gly Thr Gln His						
		385			390		395
	Asp Tyr Leu Asp His Ser Asp Ile Ile Gly Trp Thr Arg Gln Gly Val						
		405			410		415
35	Thr Gln Lys Pro Gly Ser Gly Leu Ala Ala Leu Ile Thr Asp Gly Pro						
		420			425		430
40	Gly Gly Ser Lys Trp Met Tyr Val Gly Lys Gln His Ala Gly Lys Val						
		435			440		445
	Phe Tyr Asp Leu Thr Gly Asn Arg Ser Asp Thr Val Thr Ile Asn Ser						
		450			455		460
45	Asp Gly Trp Gly Glu Phe Lys Val Asn Gly Gly Ser Val Ser Val Trp						
		465			470		475
	Val Pro Arg Lys Thr Thr Val Ser Thr Ile Ala Trp Ser Ile Thr Thr						
		485			490		495
50	Arg Pro Trp Thr Asp Gln Phe Val Arg Trp Thr Glu Pro Arg Leu Val						
		500			505		510
55	Ala Trp						

(2) INFORMATION FOR SEQ ID NO: 4:

(1) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 483 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(11) MOLECULE TYPE: protein

(12) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

Ala	Asn	Leu	Asn	Gly	Thr	Leu	Met	Gln	Tyr	Phe	Glu	Trp	Tyr	Met	Pro
1			5					10						15	
Asn	Asp	Gly	Gln	His	Trp	Arg	Arg	Leu	Gln	Asn	Asp	Ser	Ala	Tyr	Leu
		20					25						30		
Ala	Glu	His	Gly	Ile	Thr	Ala	Val	Trp	Ile	Pro	Pro	Ala	Tyr	Lys	Gly
		35					40						45		
Thr	Ser	Gln	Ala	Asp	Val	Gly	Tyr	Gly	Ala	Tyr	Asp	Leu	Tyr	Asp	Leu
		50				55					60				
Gly	Glu	Phe	His	Gln	Lys	Gly	Thr	Val	Arg	Thr	Lys	Tyr	Gly	Thr	Lys
		65			70				75					80	
Gly	Glu	Leu	Gln	Ser	Ala	Ile	Lys	Ser	Leu	His	Ser	Arg	Asp	Ile	Asn
			85					90						95	
Val	Tyr	Gly	Asp	Val	Val	Ile	Asn	His	Lys	Gly	Gly	Ala	Asp	Ala	Thr
			100					105						110	
Glu	Asp	Val	Thr	Ala	Val	Glu	Val	Asp	Pro	Ala	Asp	Arg	Asn	Arg	Val
		115					120					125			
Ile	Ser	Gly	Glu	His	Leu	Ile	Lys	Ala	Trp	Thr	His	Phe	His	Phe	Pro
		130					135					140			
Gly	Arg	Gly	Ser	Thr	Tyr	Ser	Asp	Phe	Lys	Trp	His	Trp	Tyr	His	Phe
		145			150				155					160	
Asp	Gly	Thr	Asp	Trp	Asp	Glu	Ser	Arg	Lys	Leu	Asn	Arg	Ile	Tyr	Lys
			165					170						175	
Phe	Gln	Gly	Lys	Ala	Trp	Asp	Trp	Glu	Val	Ser	Asn	Glu	Asn	Gly	Asn
			180				185							190	
Tyr	Asp	Tyr	Leu	Met	Tyr	Ala	Asp	Ile	Asp	Tyr	Asp	His	Pro	Asp	Val
		195					200					205			
Ala	Ala	Glu	Ile	Lys	Arg	Trp	Gly	Thr	Trp	Tyr	Ala	Asn	Glu	Leu	Gln
		210					215					220			
Leu	Asp	Gly	Phe	Arg	Leu	Asp	Ala	Val	Lys	His	Ile	Lys	Phe	Ser	Phe
		225				230					235				240

Leu Arg Asp Trp Val Asn His Val Arg Glu Lys Thr Gly Lys Glu Met
 245 250 255
 5 Phe Thr Val Ala Glu Tyr Trp Gln Asn Asp Leu Gly Ala Leu Glu Asn
 260 265 270
 Tyr Leu Asn Lys Thr Asn Phe Asn His Ser Val Phe Asp Val Pro Leu
 275 280 285
 10 His Tyr Gln Phe His Ala Ala Ser Thr Gln Gly Gly Gly Tyr Asp Met
 290 295 300
 Arg Lys Leu Leu Asn Gly Thr Val Val Ser Lys His Pro Leu Lys Ser
 305 310 315 320
 15 Val Thr Phe Val Asp Asn His Asp Thr Gln Pro Gly Gln Ser Leu Glu
 325 330 335
 Ser Thr Val Gln Thr Trp Phe Lys Pro Leu Ala Tyr Ala Phe Ile Leu
 340 345 350
 Thr Arg Glu Ser Gly Tyr Pro Gln Val Phe Tyr Gly Asp Met Tyr Gly
 355 360 365
 25 Thr Lys Gly Asp Ser Gln Arg Glu Ile Pro Ala Leu Lys His Lys Ile
 370 375 380
 Glu Pro Ile Leu Lys Ala Arg Lys Gln Tyr Ala Tyr Gly Ala Gln His
 385 390 395 400
 30 Asp Tyr Phe Asp His His Asp Ile Val Gly Trp Thr Arg Gln Gly Asp
 405 410 415
 Ser Ser Val Ala Asn Ser Gly Leu Ala Ala Leu Ile Thr Asp Gly Pro
 420 425 430
 Gly Gly Ala Lys Arg Met Tyr Val Gly Arg Gln Asn Ala Gly Glu Thr
 435 440 445
 40 Trp His Asp Ile Thr Gly Asn Arg Ser Glu Pro Val Val Ile Asn Ser
 450 455 460
 Glu Gly Trp Gly Glu Phe His Val Asn Gly Gly Ser Val Ser Ile Tyr
 465 470 475 480
 45 Val Gln Arg

(2) INFORMATION FOR SEQ ID NO: 5:

- 50 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 480 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear
 55 (ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

5	Val Asn Gly Thr Leu Met Gln Tyr Phe Glu Trp Tyr Thr Pro Asn Asp	1	5	10	15
	Gly Gln His Trp Lys Arg Leu Gln Asn Asp Ala Glu His Leu Ser Asp	20	25	30	
10	Ile Gly Ile Thr Ala Val Trp Ile Pro Pro Ala Tyr Lys Gly Leu Ser	35	40	45	
	Gln Ser Asp Asn Gly Tyr Gly Pro Tyr Asp Leu Tyr Asp Leu Gly Glu	50	55	60	
15	Phe Gln Gln Lys Gly Thr Val Arg Thr Lys Tyr Gly Thr Lys Ser Glu	65	70	75	80
	Leu Gln Asp Ala Ile Gly Ser Leu His Ser Arg Asn Val Gln Val Tyr	85	90	95	
20	Gly Asp Val Val Leu Asn His Lys Ala Gly Ala Asp Ala Thr Gln Asp	100	105	110	
25	Val Thr Ala Val Glu Val Asn Pro Ala Asn Arg Asn Gln Glu Thr Ser	115	120	125	
	Glu Glu Tyr Gln Ile Lys Ala Trp Thr Asp Phe Arg Phe Pro Gly Arg	130	135	140	
30	Gly Asn Thr Tyr Ser Asp Phe Lys Trp His Trp Tyr His Phe Asp Gly	145	150	155	160
	Ala Asp Trp Asp Glu Ser Arg Lys Ile Ser Arg Ile Phe Lys Phe Arg	165	170	175	
35	Gly Glu Gly Lys Ala Trp Asp Trp Glu Val Ser Ser Glu Asn Gly Asn	180	185	190	
40	Tyr Asp Tyr Leu Met Tyr Ala Asp Val Asp Tyr Asp His Pro Asp Val	195	200	205	
	Val Ala Glu Thr Lys Lys Trp Gly Ile Trp Tyr Ala Asn Gln Leu Ser	210	215	220	
45	Leu Asp Gly Phe Arg Ile Asp Ala Ala Lys His Ile Lys Phe Ser Phe	225	230	235	240
	Leu Arg Asp Trp Val Gln Ala Val Arg Gln Ala Thr Gly Lys Glu Met	245	250	255	
50	Phe Thr Val Ala Glu Tyr Trp Gln Asn Asn Ala Gly Lys Leu Glu Asn	260	265	270	
55	Tyr Leu Asn Lys Thr Ser Phe Asn Gln Ser Val Phe Asp Val Pro Leu	275	280	285	

10

His Phe Asn Leu Gln Ala Ala Ser Ser Gln Gly Gly Gly Tyr Asp Met
 290 295 300
 5 Arg Arg Leu Leu Asp Gly Thr Val Val Ser Arg His Pro Glu Lys Ala
 305 310 315 320
 Val Thr Phe Val Glu Asn His Asp Thr Gln Pro Gly Gln Ser Leu Glu
 325 330 335
 10 Ser Thr Val Gln Thr Trp Phe Lys Pro Leu Ala Tyr Ala Phe Ile Leu
 340 345 350
 15 Thr Arg Glu Ser Gly Tyr Pro Gln Val Phe Tyr Gly Asp Met Tyr Gly
 355 360 365
 Thr Lys Gly Thr Ser Pro Lys Glu Ile Pro Ser Leu Lys Asp Asn Ile
 370 375 380
 20 Glu Pro Ile Leu Lys Ala Arg Lys Glu Tyr Ala Tyr Gly Pro Gln His
 385 390 395 400
 Asp Tyr Ile Asp His Pro Asp Val Ile Gly Trp Thr Arg Glu Gly Asp
 405 410 415
 25 Ser Ser Ala Ala Lys Ser Gly Leu Ala Ala Leu Ile Thr Asp Gly Pro
 420 425 430
 Gly Gly Ser Lys Arg Met Tyr Ala Gly Leu Lys Asn Ala Gly Glu Thr
 435 440 445
 30 Trp Tyr Asp Ile Thr Gly Asn Arg Ser Asp Thr Val Lys Ile Gly Ser
 450 455 460
 35 Asp Gly Trp Gly Glu Phe His Val Asn Asp Gly Ser Val Ser Ile Tyr
 465 470 475 480
 40 (2) INFORMATION FOR SEQ ID NO: 6:
 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 485 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear
 45 (ii) MOLECULE TYPE: peptide
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:
 50 His His Asn Gly Thr Asn Gly Thr Met Met Gln Tyr Phe Glu Trp Tyr
 1 5 10 15
 Leu Pro Asn Asp Gly Asn His Trp Asn Arg Leu Asn Ser Asp Ala Ser
 20 25 30

	Asn	Leu	Lys	Ser	Lys	Gly	Ile	Thr	Ala	Val	Trp	Ile	Pro	Pro	Ala	Trp
	35						40					45				
5	Lys	Gly	Ala	Ser	Gln	Asn	Asp	Val	Gly	Tyr	Gly	Ala	Tyr	Asp	Leu	Tyr
	50				55						60					
	Asp	Leu	Gly	Glu	Phe	Asn	Gln	Lys	Gly	Thr	Val	Arg	Thr	Lys	Tyr	Gly
	65				70					75						80
10	Thr	Arg	Ser	Gln	Leu	Gln	Ala	Ala	Val	Thr	Ser	Leu	Lys	Asn	Asn	Gly
				85						90						95
	Ile	Gln	Val	Tyr	Gly	Asp	Val	Val	Met	Asn	His	Lys	Gly	Gly	Ala	Asp
15				100					105					110		
	Ala	Thr	Glu	Met	Val	Arg	Ala	Val	Glu	Val	Asn	Pro	Asn	Asn	Arg	Asn
				115					120							
20	Gln	Glu	Val	Thr	Gly	Glu	Tyr	Thr	Ile	Glu	Ala	Trp	Thr	Arg	Phe	Asp
	130						135						140			
	Phe	Pro	Gly	Arg	Gly	Asn	Thr	His	Ser	Ser	Phe	Lys	Trp	Arg	Trp	Tyr
	145				150						155					160
25	His	Phe	Asp	Gly	Val	Asp	Trp	Asp	Gln	Ser	Arg	Arg	Leu	Asn	Asn	Arg
				165					170							175
	Ile	Tyr	Lys	Phe	Arg	Gly	His	Gly	Lys	Ala	Trp	Asp	Trp	Glu	Val	Asp
30				180					185					190		
	Thr	Glu	Asn	Gly	Asn	Tyr	Asp	Tyr	Leu	Met	Tyr	Ala	Asp	Ile	Asp	Met
				195					200				205			
35	Asp	His	Pro	Glu	Val	Val	Asn	Glu	Leu	Arg	Asn	Trp	Gly	Val	Trp	Tyr
	210						215						220			
	Thr	Asn	Thr	Leu	Gly	Leu	Asp	Gly	Phe	Arg	Ile	Asp	Ala	Val	Lys	His
	225					230					235					240
40	Ile	Lys	Tyr	Ser	Phe	Thr	Arg	Asp	Trp	Ile	Asn	His	Val	Arg	Ser	Ala
				245						250						255
	Thr	Gly	Lys	Asn	Met	Phe	Ala	Val	Ala	Glu	Phe	Trp	Lys	Asn	Asp	Leu
45				260					265						270	
	Gly	Ala	Ile	Glu	Asn	Tyr	Leu	Gln	Lys	Thr	Asn	Trp	Asn	His	Ser	Val
				275					280				285			
50	Phe	Asp	Val	Pro	Leu	His	Tyr	Asn	Leu	Tyr	Asn	Ala	Ser	Lys	Ser	Gly
	290						295						300			
	Gly	Asn	Tyr	Asp	Met	Arg	Asn	Ile	Phe	Asn	Gly	Thr	Val	Val	Gln	Arg
	305					310					315					320
55	His	Pro	Ser	His	Ala	Val	Thr	Phe	Val	Asp	Asn	His	Asp	Ser	Gln	Pro

	325	330	335
	Glu Glu Ala Leu Glu Ser Phe Val Glu Glu Trp Phe Lys Pro Leu Ala		
	340	345	350
5	Tyr Ala Leu Thr Leu Thr Arg Glu Gln Gly Tyr Pro Ser Val Phe Tyr		
	355	360	365
10	Gly Asp Tyr Tyr Gly Ile Pro Thr His Gly Val Pro Ala Met Arg Ser		
	370	375	380
	Lys Ile Asp Pro Ile Leu Glu Ala Arg Gln Lys Tyr Ala Tyr Gly Lys		
	385	390	400
15	Gln Asn Asp Tyr Leu Asp His His Asn Ile Ile Gly Trp Thr Arg Glu		
	405	410	415
	Gly Asn Thr Ala His Pro Asn Ser Gly Leu Ala Thr Ile Met Ser Asp		
20	420	425	430
	Gly Ala Gly Gly Ser Lys Trp Met Phe Val Gly Arg Asn Lys Ala Gly		
	435	440	445
25	Gln Val Trp Ser Asp Ile Thr Gly Asn Arg Thr Gly Thr Val Thr Ile		
	450	455	460
	Asn Ala Asp Gly Trp Gly Asn Phe Ser Val Asn Gly Gly Ser Val Ser		
	465	470	475
30	Ile Trp Val Asn Lys		
	485		

(2) INFORMATION FOR SEQ ID NO: 7:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 485 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: peptide
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

	His His Asn Gly Thr Asn Gly Thr Met Met Gln Tyr Phe Glu Trp Tyr		
	1	5	10
45	Leu Pro Asn Asp Gly Asn His Trp Asn Arg Leu Arg Asp Asp Ala Ala		
	20	25	30
	Asn Leu Lys Ser Lys Gly Ile Thr Ala Val Trp Ile Pro Pro Ala Trp		
	35	40	45
50	Lys Gly Thr Ser Gln Asn Asp Val Gly Tyr Gly Ala Tyr Asp Leu Tyr		
	50	55	60
55	Asp Leu Gly Glu Phe Asn Gln Lys Gly Thr Val Arg Thr Lys Tyr Gly		
	65	70	75
			80

Thr Arg Asn Gln Leu Gln Ala Ala Val Thr Ser Leu Lys Asn Asn Gly
 85 90 95
 5 Ile Gln Val Tyr Gly Asp Val Val Met Asn His Lys Gly Gly Ala Asp
 100 105 110
 Gly Thr Glu Ile Val Asn Ala Val Glu Val Asn Arg Ser Asn Arg Asn
 115 120 125
 10 Gln Glu Thr Ser Gly Glu Tyr Ala Ile Glu Ala Trp Thr Lys Phe Asp
 130 135 140
 Phe Pro Gly Arg Gly Asn Asn His Ser Ser Phe Lys Trp Arg Trp Tyr
 145 150 155 160
 15 His Phe Asp Gly Thr Asp Trp Asp Gln Ser Arg Gln Leu Gln Asn Lys
 165 170 175
 20 Ile Tyr Lys Phe Arg Gly Thr Gly Lys Ala Trp Asp Trp Glu Val Asp
 180 185 190
 Thr Glu Asn Gly Asn Tyr Asp Tyr Leu Met Tyr Ala Asp Val Asp Met
 195 200 205
 25 Asp His Pro Glu Val Ile His Glu Leu Arg Asn Trp Gly Val Trp Tyr
 210 215 220
 Thr Asn Thr Leu Asn Leu Asp Gly Phe Arg Ile Asp Ala Val Lys His
 225 230 235 240
 30 Ile Lys Tyr Ser Phe Thr Arg Asp Trp Leu Thr His Val Arg Asn Thr
 245 250 255
 35 Thr Gly Lys Pro Met Phe Ala Val Ala Glu Phe Trp Lys Asn Asp Leu
 260 265 270
 Gly Ala Ile Glu Asn Tyr Leu Asn Lys Thr Ser Trp Asn His Ser Val
 275 280 285
 40 Phe Asp Val Pro Leu His Tyr Asn Leu Tyr Asn Ala Ser Asn Ser Gly
 290 295 300
 Gly Tyr Tyr Asp Met Arg Asn Ile Leu Asn Gly Ser Val Val Gln Lys
 305 310 315 320
 45 His Pro Thr His Ala Val Thr Phe Val Asp Asn His Asp Ser Gln Pro
 325 330 335
 50 Gly Glu Ala Leu Gln Ser Phe Val Gln Gln Trp Phe Lys Pro Leu Ala
 340 345 350
 Tyr Ala Leu Val Leu Thr Arg Glu Gln Gly Tyr Pro Ser Val Phe Tyr
 355 360 365
 55

Gly Asp Tyr Tyr Gly Ile Pro Thr His Gly Val Pro Ala Met Lys Ser
 370 375 380
 5 Lys Ile Asp Pro Leu Leu Gln Ala Arg Gln Thr Phe Ala Tyr Gly Thr
 385 390 395 400
 Gln His Asp Tyr Phe Asp His His Asp Ile Ile Gly Trp Thr Arg Glu
 405 410 415
 10 Gly Asn Ser Ser His Pro Asn Ser Gly Leu Ala Thr Ile Met Ser Asp
 420 425 430
 Gly Pro Gly Gly Asn Lys Trp Met Tyr Val Gly Lys Asn Lys Ala Gly
 435 440 445
 15 Gln Val Trp Arg Asp Ile Thr Gly Asn Arg Thr Gly Thr Val Thr Ile
 450 455 460
 20 Asn Ala Asp Gly Trp Gly Asn Phe Ser Val Asn Gly Gly Ser Val Ser
 465 470 475 480
 Val Trp Val Lys Gln
 485
 25 (2) INFORMATION FOR SEQ ID NO: 8:
 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 485 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: single
 30 (D) TOPOLOGY: linear
 (ii) MOLECULE TYPE: peptide
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:
 His His Asn Gly Thr Asn Gly Thr Met Met Gln Tyr Phe Glu Trp His
 35 1 5 10 15
 Leu Pro Asn Asp Gly Asn His Trp Asn Arg Leu Arg Asp Asp Ala Ser
 20 25 30
 40 Asn Leu Arg Asn Arg Gly Ile Thr Ala Ile Trp Ile Pro Pro Ala Trp
 35 40 45
 Lys Gly Thr Ser Gln Asn Asp Val Gly Tyr Gly Ala Tyr Asp Leu Tyr
 45 50 55 60
 Asp Leu Gly Glu Phe Asn Gln Lys Gly Thr Val Arg Thr Lys Tyr Gly
 65 70 75 80
 50 Thr Arg Ser Gln Leu Glu Ser Ala Ile His Ala Leu Lys Asn Asn Gly
 85 90 95
 Val Gln Val Tyr Gly Asp Val Val Met Asn His Lys Gly Gly Ala Asp
 100 105 110
 55 Ala Thr Gln Asn Val Leu Ala Val Glu Val Asn Pro Asn Asn Arg Asn

15

	115	120	125
5	Gln Glu Ile Ser Gly Asp Tyr Thr Ile Glu Ala Trp Thr Lys Phe Asp 130 135 140		
	Phe Pro Gly Arg Gly Asn Thr Tyr Ser Asp Phe Lys Trp Arg Trp Tyr 145 150 155 160		
10	His Phe Asp Gly Val Asp Trp Asp Gln Ser Arg Gln Phe Gln Asn Arg 165 170 175		
	Ile Tyr Lys Phe Arg Gly Asp Gly Lys Ala Trp Asp Trp Glu Val Asp 180 185 190		
15	Ser Gln Asn Gly Asn Tyr Asp Tyr Leu Met Tyr Ala Asp Val Asp Met 195 200 205		
20	Asp His Pro Glu Val Val Asn Glu Leu Arg Arg Trp Gly Glu Trp Tyr 210 215 220		
	Thr Asn Thr Leu Asn Leu Asp Gly Phe Arg Ile Asp Ala Val Lys His 225 230 235 240		
25	Ile Lys Tyr Ser Phe Thr Arg Asp Trp Leu Thr His Val Arg Asn Ala 245 250 255		
	Thr Gly Lys Glu Met Phe Ala Val Ala Glu Phe Trp Lys Asn Asp Leu 260 265 270		
30	Gly Ala Leu Glu Asn Tyr Leu Asn Lys Thr Asn Trp Asn His Ser Val 275 280 285		
35	Phe Asp Val Pro Leu His Tyr Asn Leu Tyr Asn Ala Ser Asn Ser Gly 290 295 300		
	Gly Asn Tyr Asp Met Ala Lys Leu Leu Asn Gly Thr Val Val Gln Lys 305 310 315 320		
40	His Pro Met His Ala Val Thr Phe Val Asp Asn His Asp Ser Gln Pro 325 330 335		
	Gly Glu Ser Leu Glu Ser Phe Val Gln Glu Trp Phe Lys Pro Leu Ala 340 345 350		
45	Tyr Ala Leu Ile Leu Thr Arg Glu Gln Gly Tyr Pro Ser Val Phe Tyr 355 360 365		
50	Gly Asp Tyr Tyr Gly Ile Pro Thr His Ser Val Pro Ala Met Lys Ala 370 375 380		
	Lys Ile Asp Pro Ile Leu Glu Ala Arg Gln Asn Phe Ala Tyr Gly Thr 385 390 395 400		
55	Gln His Asp Tyr Phe Asp His His Asn Ile Ile Gly Trp Thr Arg Glu 405 410 415		

	Gly Asn Thr Thr His Pro Asn Ser Gly Leu Ala Thr Ile Met Ser Asp	
	420 425 430	
5	Gly Pro Gly Gly Glu Lys Trp Met Tyr Val Gly Gln Asn Lys Ala Gly	
	435 440 445	
	Gln Val Trp His Asp Ile Thr Gly Asn Lys Pro Gly Thr Val Thr Ile	
10	450 455 460	
	Asn Ala Asp Gly Trp Ala Asn Phe Ser Val Asn Gly Gly Ser Val Ser	
	465 470 475 480	
	Ile Trp Val Lys Arg	
15	485	
	(2) INFORMATION FOR SEQ ID NO: 9:	
	(i) SEQUENCE CHARACTERISTICS:	
	(A) LENGTH: 1455 base pairs	
20	(B) TYPE: nucleic acid	
	(C) STRANDEDNESS: single	
	(D) TOPOLOGY: linear	
	(ii) MOLECULE TYPE: DNA (genomic)	
	(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:	
25	CATCATAATG GAACCAATGG TACTATGATG CAATATTTCG AATGGTATTT GCCAAATGAC	60
	GGGAATCAAT GGAACAGGTT GAGGGATGAC GCAGCTAACT TAAAGAGTAA ACGGATAACA	120
30	GCTGTATGGA TCCACCTGCG ATUGAAGGGG ACTTCCAGA ATGATGTAGG TTATGGAGCC	180
	TATGATTTAT ATGATCTTGG AGAGTTTAAAC CAGAAGGGGA CGETTCGTAC AAAATATGGA	240
	ACACGCAACC AGCTACAGGC TCGGGTGACC TCTTTAAAAA ATAACGGCAT TCAGGTATAT	300
35	GGTGATGTCC TCATGAATCA TAAAGGTGGA GCAGATGGTA CGGAAATTGT AATGCGGGTA	360
	GAAGTGAATC GGAGCAACCG AAACCAGGAA ACCTCAGGAG AGTATGCAAT AGAAGCGGTG	420
40	ACAAAGTTTG ATTTCCTGCG AAGAGGAAAT AACCAATCCA GCTTTAAGTG GCGCTGGTAT	480
	CAATTTTGATG GGACAGATTG GGATCACTCA CGCCAGCTTC AAAACAAAAT ATATATAATTC	540
	AGGGGAACAG GCAAGGCCTG GGACTGGGAA GTCGATACAG AGAATGBCAA CTATGACTAT	600
45	CTTATGTATG CAGACGTGGA TATGGATCAC CCAGAAATAA TACATGAACAT TAGAAACTGG	660
	GGAGTGTGGT ATACGAATAC ACTGAACCTT GATGGATTTA GAATAGATGC AGTGAACAT	720
50	ATAAAATATA GCTTTACGAG AGATTGGCTT ACACATGTGC GTAACACCAC AGGTAAACCA	780
	ATCTTTGCAG TGGCTGAGTT TTGGAAAAAT GACCTTGGTG CAATTGAAAA CTATTTGAAT	840
	AAAACAAGTT GGAATCACTC GGTGTTTGAT GTTCTCTCC ACTATAATTT GTACAATGCA	900
55		

	TCTAATAGCG GTGCTTATTA TGATATGAGA AATATTTTAA ATGCTTCTGT GGTCACAAAA	960
	CATCCAACAC ATGCCGTTAC TTTTGTGAT AACCATGATT CTCAGCCCCG GGAAGCATTG	1020
5	GAATCCTTTG TTCAACAATG GTTTAAACCA CTTGCATATG CATTTGGTCTT GACAAGGGAA	1080
	CAAGGTATAC CTTCGCTATT TTATGGGGAT TACTACGGTA TCCCAACCCA TGCTGTTCGG	1140
	GCTATGAAT CTAAATAGA CCTCTTCTG CAGGCACGTC AAACCTTTGC CTATGGTACG	1200
10	CAGCATGATT ACTTTGATCA TCATGATATT ATCGGTGGGA CAAGAGAGGG AAATAGCTCC	1260
	CATCCAATTT CAGGCCTTGC CACCATTATG TCAGATGGTC CAGGTGGTAA CAAATGGATG	1320
15	TATGTGGGGA AAAATAAGC GGGACAAGTT TGGAGAGATA TTACCGGAAA TAGGACAGGC	1380
	ACCGTCACAA TTAATGCAGA CGGATGGGGT AATTCTCTG TTAATGGAGG GTCCGTTTCG	1440
	GTTCGGCTGA AGCAA	1455
20	(2) INFORMATION FOR SEQ ID NO: 10:	
	(i) SEQUENCE CHARACTERISTICS:	
	(A) LENGTH: 1455 base pairs	
	(B) TYPE: nucleic acid	
25	(C) STRANDEDNESS: single	
	(D) TOPOLOGY: linear	
	(ii) MOLECULE TYPE: DNA (genomic)	
	(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:	
30	CATCATAATG GGACAAATGG GACGATGATG CAATACITTG AATGGCACTT GCCTAATGAT	60
	GGGAATCACT GGAATAGATT AAGAGATGAT GCTAGTAATC TAAGMAATAG AGGTATAACC	120
	GCTATTGGGA TTCCGCTTGC CTGGAAAGGG ACTTCGCAA ATGATGTGGG GTATGGAGCC	180
35	TATGATCTTT ATGATTTAGG GGAATTTAAT CAAAAGGGGA CGTTCGTAC TAAGTATGGG	240
	ACACGTAGTC AATTTGGATC TGCCATCCAT GCITTAAGA ATAATGGCGT TCAAGTTTAT	300
40	GGGGATGTAG TGAAGAACCA TAAAGGAGGA GCTGATGCTA CAGAAAACGT TCTTGTCTGC	360
	GAGGTGAATC CAAATAACCG GAATCAAGAA ATATCTGGGG ACTACACAAAT TGAGCGCTTG	420
	ACTAAGTTTG ATTTTCCAGG GAGGGTAAT ACATACTCAG ACITTTAAATG GCCTTGTAT	480
45	CATTTCCGAT GTGTAGATT GGATCAATCA CGACRATTL AAAATCGTAT CTACAAATTC	540
	CGAGGTGATG GTAAGGCATG GGATGGGAA GTAGATTCCG AAAATGGAAA TTATGATTAT	600
50	TTAATGTATG CAGATGTAGA TATGGATCAT CCGGAGGTAG TAAATGAGCT TAGAAGATGG	660
	GGAGAATGGT ATACAATAC ATTAATCTTT GATGGATTTA GGATCGATGC GGTGAAGCAT	720
	ATTAAATATA GCTTTACACG TGATTGGTTG ACCCATGTAA GAAACGCAC GGGAAAAGAA	780
55		

	APGTTTGGTG TTGCTGAATT TTGGAAAAAT GATTTAGGTG CCTTGGAGAA CTATTAAAT	840
	AAAACAACT GGAATCATTG TGTCTTGAT GTCCCCCTTC ATTATAATCT TTATAACGCG	900
5	TCAANTAGTG GAGGCACTA TGACATGGCA AACTTCTTA ATGGAACGGT TGTCAAAAG	960
	CATCCAATCG ATGCCGTAAC TTTTGTGGAT AATCAOGATT CTCAACCTGG GGAATCATT	1020
	GAATCATTG TACAAGAATG GTTTAAGCCA CTGCTTATG CCGTATTTT AACAGAGAA	1080
10	CAAGGGTATC CCTCGCTCTT CTATGGTAC TACTATGGAA TTCCAACACA TAGTGTCCCA	1140
	GCAATGAAG CCAAGATTGA TCCAATCTTA GAGGCGCGTC AAAATTTTGC ATATGGAACA	1200
15	CAACATGATT ATTTTGACCA TCATAATATA ATCGGATGGA CACGTGAAGG AAATACCAAG	1260
	CATCCCAATT CAGGACTTGC GACTATCATG TCGGATGGGC CAGGGGAGGA GAATGGATG	1320
	TACGTAGGCG AAAATAAAGC AGGTCAAGTT TGGCATGACA TAACTGGAAA TAAACAGGA	1380
20	ACAGTTACGA TCAATGCAGA TGGATGGGCT AATTTTTTCA TAAATGGAGG ATCTGTTTCC	1440
	ATTTGGGTGA AACGA	1455
25	(2) INFORMATION FOR SEQ ID NO: 11:	
	(1) SEQUENCE CHARACTERISTICS:	
	(A) LENGTH: 1548 base pairs	
	(B) TYPE: nucleic acid	
30	(C) STRANDEDNESS: single	
	(D) TOPOLOGY: linear	
	(1.1) MOLECULE TYPE: DNA (genomic)	
	(1.1) SEQUENCE DESCRIPTION: SEQ ID NO: 11:	
35	GCCGACCGT TTAACGGCAC CATGATCCAG TATTTTGAAT GGTACTTGCC GGTATGCGC	60
	ACGTTATGGA CCAAGTGGC CAATGAAGC AACACTTAT CAGCCTTGG CATCACCGCT	120
	CTTTGGCTGC CGCCCGCTTA CAAAGGAACA AGCCGACGCG ACGTAGGCTA CGGAGTATAC	180
40	GACTTGTATG ACCTCGGCGA ATTCAATCAA AAGGGACCG TCCGCACAAA ATACGGAACA	240
	AAGCTCAAT ATCTTCAAGC CATTCAAGC GCCACGCGC CTGGAATGCA AGGTACGCC	300
45	GATGTCGTGT TGCACATAA AGGCGGCGCT GACGGGACGG AATGGGTGGA CGCGGTCGAA	360
	GTCAATCCGT CGACCGCAA CCAAGAAATC TCGGGCACT ATCAATCCA AGCATGGAGC	420
	AAATTTGATT TTCCCGGGCG GGGCAACACC TACTCCAGCT TTAAGTGGCG CTGGTACCAT	480
50	TTTGACGGCG TTGATTGGGA CGAAGCGGA AAATTGAGCC GCATTTACAA ATTCGCGGCG	540
	ATCGGCAAG CGTGGGATTG GGAAGTAGAC ACGGAAAACG GAAGTATGA CTACTTAATG	600
55	TATGCCGACC TTGATATGGA TCATCCCGAA GTCTGACCG AGCTGAAAA CTGGGGGAAA	660

	TGGTATGTCA ACACAACGAA CATTGATGGG TTCCGGCTTG ATGCGGICAA GCATATTAAG	720
5	TTCCAGTTTT TTCTGATTG GTTGCTGTAT GTGCGTTCTC AGACTGGCAA GCGCTATTTT	780
	ACCGTCGGGG AATATTGGAG CTATGACATC AACAAATTGC ACAATTACAT TACGAAAACA	840
	GACGGAAACA TGCTTTTGT TGATGCCCGG TTACACAACA AATTTTATAC CGCTTCCAAA	900
10	TCAGGGGGCG CATTGTGATAT GCGGACGTTA ATGACCAATA CTCATGAA AGATCAACCG	960
	ACATTGGCCG TCACCTTCGT TGATAATCAT GACACCGAAC CCGGCCAAGC GCTGCAGTCA	1020
15	TGGGTCGACC CATGTTCAA ACCGTTGGCT TACGCGTTTA TTCTAACTCG GCAGGAAGGA	1080
	TACCGCTGCG TCTTTTATGG TGACTATTAT GGCATTCCAC AATATAACAT TCCTTCGCTG	1140
	AAAAGCAAAA TCGATCCGCT CCTCATCGCG CGCAGGGATT ATGCTTACGG AACGCAACAT	1200
20	GATTATCTTG ATCACTCCGA CATCATCGGG TGGACAAGGG AAGGGGGCAC TGAARAACCA	1260
	GGATCCGGAG TGGCCGCACT GATCACCAGT GGGCCGGGAG GAAGCAAAAT GATGTACGTT	1320
25	GGCAACAAC ACGCTGGAAA AGTGTTCTAT GACCTTACCG GCAACCGGAG TGACACCGTC	1380
	ACCATCAACA GTGATGGATG GGGGGAAATC AAGTCAATG GCGGTTCCGT TTCGGTTTGG	1440
	GTTCCTAGAA AAACGACGCT TTCTACCATC GCTCGGCCGA TCACAACCGC ACCGTGGACT	1500
30	GGTGAATTCC TCGGTTGGAC CGAACCAAGG TTGGTGGCAT GGCCTTGA	1548
	(2) INFORMATION FOR SEQ ID NO: 12:	
	(i) SEQUENCE CHARACTERISTICS:	
35	(A) LENGTH: 1920 base pairs	
	(B) TYPE: nucleic acid	
	(C) STRANDEDNESS: single	
	(D) TOPOLOGY: linear	
	(ii) MOLECULE TYPE: DNA (genomic)	
40	(ix) FEATURE:	
	(A) NAME/KEY: CDS	
	(B) LOCATION: 421..1872	
	(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:	
45	CGGAGATTG GAAGTACAAA AATAAGCAAA AGATTGTCAA TCATGTCATG AGCCATGCGG	60
	GAGACGGAAA AATCGTCTTA ATGCACGATA TTTATGCAAC GTTCGCAGAT GCTGCTGAAG	120
	AGATTATTAA AAAGCTGAAA GCAAAAGGCT ATCAATTGGT AACTGTATCT CAGCTTGAAG	180
50	AAGTGAACAA GCAGAGAGGC TATTAATAAA ATGAGTAGAA CGGCCATATC GCGCCTTTTC	240
	TTTGGGAAGA AATATAGGG AAAATGGTAC TTGTTAAAAA TTGGAATAT TTATACAACA	300
55	TCATATGTTT CACATTGAAA GGGGAGGAGA ATCATGAAAC AACAAAAAGC GCTTTACGCC	360

	CGATTGCTGA CGCTGTTATT TGCCTCATC TTCCTGCTGC CTCATTCTGC AGCAGCGGCG	420
	GCA AAT CTT AAT GGG ACG CTG ATG CAG TAT TTT GAA TGG TAC ATG CCC	468
5	AAT GAC GGC CAA CAT TGG AGG CGT TTG CAA AAC GAC TCG GCA TAT TTG	516
	GCT GAA CAC GGT ATT ACT GCC GTC TGG ATT CCC CCG GCA TAT AAG GGA	564
10	ACG AGC CAA GCG GAT GTG GGC TAC GGT GCT TAC GAC CTT TAT GAT TTA	612
	GGG GAG TTT CAT CAA AAA GGG ACG GTT CGG ACA AAG TAC GGC ACA AAA	660
	GGA GAG CTG CAA TCT GCG ATC AAA AGT CTT CAT TCC CGC GAC ATT AAC	708
15	GTT TAC GGG GAT GTG GTC ATC AAC CAC AAA GGC GGC GCT GAT GCG ACC	756
	GAA GAT GTA ACC GCG GTT GAA GTC GAT CCC GCT GAC CGC AAC CGC GTA	804
20	ATT TCA GGA GAA CAC CTA ATT AAA GCC TGG ACA CAT TTT CAT TTT CCG	852
	GGG CGC GGC AGC ACA TAC AGC GAT TTT AAA TGG CAT TGG TAC CAT TTT	900
	GAC GGA ACC GAT TGG GAC GAG TCC CGA AAG CTG AAC CGC ATC TAT AAG	948
25	TTT CAA GGA AAG GCT TGG GAT TGG GAA GTT TCC AAT GAA AAC GGC AAC	996
	TAT GAT TAT TTG ATG TAT GCC GAC ATC GAT TAT GAC CAT CCT GAT GTC	1044
30	GCA GCA GAA ATT AAG AGA TGG GGC ACT TGG TAT GCC AAT GAA CTG CAA	1092
	TTG GAC GGT TTC CGT CTT GAT GCT GTC AAA CAC ATT AAA TTT TCT TTT	1140
	TTG CGG GAT TGG GTT AAT CAT GTC AGG GAA AAA ACG GGG AAG GAA ATG	1188
35	TTT ACG GTA GCT GAA TAT TGG CAG AAT GAC TTG GGC GCG CTG GAA AAC	1236
	TAT TTG AAC AAA ACA AAT TTT AAT CAT TCA GTG TTT GAC GTG CCG CTT	1284
40	CAT TAT CAG TTC CAT GCT GCA TCG ACA CAG GGA GGC GGC TAT GAT ATG	1332
	AGG AAA TTG CTG AAC GGT ACG CTC GTT TCC AAG CAT CCG TTG AAA TCG	1380
	GTT ACA TTT GTC GAT AAC CAT GAT ACA CAG CCG GCG CAA TCG CTT GAG	1428
45	TCG ACT GTC CAA ACA TGG TTT AAG CCG CTT GCT TAC GCT TTT ATT CTC	1476
	ACA AGG GAA FCT GGA TAC CCT CAG GTT TTC TAC GGG GAT ATG TAC GGG	1524
50	ACG AAB GGA GAC TCC CAG CGC GAA ATT CCT GCC TTG AAA CAC AAA ATT	1572
	GAA CCG ATC TTA AAA CCG ACA AAA CAG TAT GCG TAC GGA GCA CAG CAT	1620
	GAT TAT TTC GAC CAC CAT GAC ATT GTC GGC TGG ACA AGG GAA GGC GAC	1668
55		

	AGC TCG GTT GCA AAT TCA GGT TTG GCG GCA TTA ATA ACA GAC GGA CCC	1716
	GGT GGG GCA AAG CGA ATG TAT GTC GGC CGG CAA AAC GCC GGT GAG ACA	1764
5	TGG CMT GAC ATT ACC GGA AAC CGT TCG GAG CCG GTT GTC ATC AAT TCG	1812
	GAA GGC TCG GGA GAG TTT CAC GTA AAC GGC GGG TCG GTT TCA ATT TAT	1860
	GTT CAA AGA TAG AAGAGCAGAG AGGACGGATT TCCTGAAGGA AATCGGTTTT	1912
10	TTTATTTT	1920
(2) INFORMATION FOR SEQ ID NO: 12:		
15	(i) SEQUENCE CHARACTERISTICS:	
	(A) LENGTH: 2084 base pairs	
	(B) TYPE: nucleic acid	
	(C) STRANDEDNESS: single	
	(D) TOPOLOGY: linear	
20	(ii) MOLECULE TYPE: DNA (genomic)	
	(ix) FEATURE:	
	(A) NAME/KEY: CDS	
	(B) LOCATION:343..1794	
	(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:	
25	GGCCCGCACA TACGAAAAGA CTGGCTGAAA ACATTGAGCC TTGTATGACT GATGATTTGG	60
	CTGAAGAAGT GGATCGATTG TTGAGAAAA GAGAAGACC ATAAAAATAC CTCGTCTGTC	120
30	ATCAGACAGG GTATTTTTTA TGCTGTCCAG ACTGTCCGCT GTGTAAAAAT AAGGAATAAA	180
	GGGGGGTTGT TATTATTTTA CTGATATGTA AATATAATT TGTATAAGAA AATGAGAGGG	240
	AGAGGAACA TGATTCAAAA ACGAAGCGG ACAGTTTCGT TCAGACTTGT GCTTAIGTGC	300
35	ACGCTGTAT TTGTCAGTTT GCCGATTACA AAAACATCAG CC GTA AAT GGC ACG	364
	CTG ATG CAG TAT TTT GAA TGG TAT ACG CCG AAC GAC GGC CAG CAT TGG	402
40	AAA CGA TTG CAG AAT GAT GCG GAA CAT TTA TCG GAT ATC GGA ATC ACT	460
	GCC CTC TGG ATT CCT CCC GCA TAC AAA GGA TTG AGC CAA TCC GAT AAC	498
	GGA TAC GGA CDT TAT GAT TTG TAT GAT TTA GGA GAA TTC CAG CAA AAA	546
45	GGG ACG GTC AGA ACG AAA TAC GGC ACA AAA TCA GAG CTT CAA GAT GCG	594
	ATC GGC TCA CTG CMT TCC CGG AAC GTC CAA GTA TAC GGA GAT GTG GTT	642
50	TTG AAT CAT AAG GCT GGT GCT GAT GCA ACA GAA GAT GTA ACT GCC GTC	690
	GAA GTC AAT CCG GCC AAT AGA AAT CAG GAA ACT TCG GAG GAA TAT CAA	738
	ATC AAA GCG TGG ACG GAT TTT CGT TTT CCG GGC CGT GGA AAC ACG TAC	786
55		

	AGT GAT TTT AAA TGG CAT TGG TAT CAT TTC GAC GGA GCG GAC TGG GAT	934
	GAA TCC CGG AAG ATC AGC CGC ATC TTT AAG TTT CGT GGG GAA GGA AAA	982
5	GCG TGG GAT TGG GAA GTA TCA AGT GAA AAC GGC AAC TAT GAC TAT TTA	930
	ATG TAT GGT GAT GTT GAC TAC GAC CAC CCT GAT GTC GTG GCA GAG ACA	978
	AAA AAA TGG GGT ATC TGG TAT GCG AAT GAA CTG TCA TTA GAC GGC TTC	1026
10	CGT ATT GAT GCC GGC AAA CAT ATT AAA TTT TCA TTT CTG CGT GAT TGG	1074
	GTT CAG GCG GTC AGA CAG GCG ACG GGA AAA GAA ATG TTT ACG GTT GCG	1122
15	GAG TAT TGG CAG AAT AAT GCC GGG AAA CTC GAA AAC TAC TTG AAT AAA	1170
	ACA AGC TTT AAT CAA TCC GTG TTT GAT GTT CCG CTT CAT TTC AAT TTA	1218
	CAG GCG GCT TCC TCA CAA GGA GGC GGA TAT GAT ATG AGG GGT TTG CTG	1266
20	GAC GGT ACC GTT GTG TCC AGG CAT CCG GAA AAG GCG GTT ACA TTT GTT	1314
	GAA AAT CAT GAC ACA CAG CCG GGA CAG TCA TTG GAA TCG ACA GTC CAA	1362
25	ACT TGG TTT AAA CCG CTT GCA TAC GCC TTT ATT TTG ACA AGA GAA TCC	1410
	GGT TAT CCT CAG GTG TTC TAT GGG GAT ATG TAC GGG ACA AAA GGG ACA	1458
	TCG CCA AAG GAA ATT CCC TCA CTG AAA GAT AAT ATA GAG CCG ATT TTA	1506
30	AAA GCG CGT AAG GAG TAC GCA TAC GGG CCC CAG CAC GAT TAT ATT GAC	1554
	CAC CCG GAT GTG ATC GGA TGG ACG AGG GAA GGT GAC AGC TCC GCC GCC	1602
35	AAA TCA GGT TTG GCC GCT TTA ATC ACG GAC GGA CCC GGC GGA TCA AAG	1650
	CGG ATG TAT GCC GGC CTG AAA AAT GCC GGC GAG ACA TGG TAT GAC ATA	1698
	ACG GGC AAC CGT TCA GAT ACT GTA AAA ATC GGA TCT GAC GGC TGG GGA	1746
40	GAG TTT CAT GTA AAC GAT GGG TCC GTC TCC ATT TAT GTT CAG AAA TAA	1794
	GGTAATAAAA AAACACCICC AAGCTGAGTC CGGGTATCAG CTTGGAGGTG CGTTTATTTT	1854
45	TTCAAGCCGTA TGACAAGTTC GGCATCAGGT GTGACAAATA CGGTATGCTG GCTGTCATAG	1914
	GTGACAAATC CCGGTTTTGC GCGGTTTGCC TTTTCACAT GTCTGATTTT TGTATAATCA	1974
	ACAGGCACGG AGCCGGRATC TTTGCUCTTG GAAAAATAAG CGGCGATCGT AGCTGCTTCC	2034
50	AATATGGATT GTTCATCGGG ATCGCTGCTT TTAATCACAA CGTGGGATCC	2084

(2) INFORMATION FOR SEQ ID NO: 13:

(1) SEQUENCE CHARACTERISTICS:

	(A) LENGTH: 1455 base pairs	
	(B) TYPE: nucleic acid	
	(C) STRANDEDNESS: single	
	(D) TOPOLOGY: linear	
5	(ii) MOLECULE TYPE: DNA (genomic)	
	(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:	
	CATCATAATG GACCAATGG TACTATGATG CATTATTTCG AATGGIATTT GCCAAATGAC	60
10	GGGAATCATT GGAACAGGTT GAGGGATGAC GCAGCTAACT TAAAGAGTAA AGGGATAACA	120
	GCTGTATGGA TCCCACTGCG ATGGAAGGGG ACTTCCGAGA ATGATGTAGG TTATGGAGCC	180
15	TATGATTTAT ATGATCTTGG AGAGTTTAAC CAGAAGGGGA CGGTTCTGTC AAAATATGGA	240
	ACACGCAACC AGCTACAGGC TCGGTGACC TCTTTAAAAA ATAACGGCAT TCAGTATAT	300
	GGTGATGTGG TCATGAATCA TAAAGGTGGA GCAGATGGTA CGGAATTGTG AAATACGGTA	360
20	GAAGTGAATC GGAGCAACCG AAACCAGGAA ACCTCAGGAG AGTATGCAAT AGAAGCGTGG	420
	ACAAAGTTTG ATTTTCTGCG AAGAGGAAAT AACCATTCCT GCTTTAGTGG GCGCTGCTAT	480
25	CATTTTGATG GGACAGATTG GGATCAGTCA CGCCAGCTTC AAAACAAAT ATATAAATTC	540
	AGGGGAACAG GCAAGGCCCTG GGACTGGGAA CTCGATACAG AGAATGGCAA CTATGACTAT	600
	CTTATGTATG CAGACCTGGA TATGATCAG CCAAGAGTAA TACATGAAT TAGAACTGG	660
30	GGAGTGTGGT ATACGANTAC ACTGAACCTT GATGGATTTA GAATAGATGC AGTGAACAT	720
	ATAAAATATA GCTTTACGAG AGATTGGCTT ACACATGTGC GTACACCCAC AGGTAACCA	780
35	ATGTTTGCAG TGGCTGAGTT TTGGAAAAAT GACCTTGGTG CAATTGAAAA CTATTTGAAT	840
	AAAACAAGTT GGAATCACTC GGTGTTTGAT GTTCTCTCC ACTATAATT GTACAATGCA	900
	TCTAATAGCG GTGGTTATTA TGATATGAGA AATATTTTAA ATGTTCTGTG GTGCAAAAA	960
40	CATCCAAACG ATGCCGTTAC TTTTGTGAT AAACATGATT CTCAGCCCCG GGAGCATTTG	1020
	GAATCCTTTG TTCAACAATG GTTTAAACCA CTTCGATATG CATGCTTCTT GACAAGGGAA	1080
45	CAAGGTTATC CTTCCGTTAT TTATGGGGAT TACTACGGTA TCCCAACCCA TGGTGTCCCG	1140
	GCTATGAATC CTAAATAGA CCTCTTCTG CAGGCACGTC AAACCTTTTC CTATGGTACG	1200
	CAGCATGATT ACITTGATCA TCATGATATT ATCGTTTGG CAAGAGAGGG AAATAGCTCC	1260
50	CATCCAAATT CAGGCCTTGC CACCATTATG TCAGATGGTC CAGGTGGTAA CAAATGGATC	1320
	TATGTGGGGA AAAATAAAGC GGGACAGTT TGGAGAGATA TTACCGGAAA TAGGACAGGC	1380
55	ACCGTCACAA TTAATGCAGA CGGATGGGGT AATTTCTCTG TTAATGGAGG GTCCGTTTCG	1440

GTTTGGGTGA AGCAA

1455

(2) INFORMATION FOR SEQ ID NO: 14:

(i) SEQUENCE CHARACTERISTICS:

- 5 (A) LENGTH: 1455 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

10 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

	CATCATATG GGACAATGG GACGATGATG CAATACCTTG AATGGCACTT GCCTAATGAT	60
	GGGAATCACT GGAATAGATT AAGAGATGAT GCTAGTAATC TAAGAAATAG AGGTATAACC	120
15	GCTATTTCGA TTCCGCCGCG CTGGAAAGGG ACTTCGCAAA ATGATGTGGG GTATCGAGCC	180
	TATGATCTTT ATGATTTAGG GGAATTTAAT CAAAAGGGGA CGGTCGTAC TAAGTATGGG	240
20	ACACGTAGTC AATTGGAGTC TGCCATCCAT GCCTTAAAGA ATAATGGCGT TCAAGTTTAT	300
	GGGGATGATG TGATGAACCA TAAAGGAGGA GCTGATGCTA CAGAAACGT TCTTGCTGTC	360
	GAGGTGAATC CAATAACCG GAATCAGGAA ATATCTGGGG ACTACADAAT TGAGGCTTGG	420
25	ACTAAGTTTG ATTTTCCAGG GAGGGGTAAAT ACATACTCAG ACTTTAAATG GCCTTGGTAT	480
	CATTTCGATG GTGTAGATTG GGATCATCA CGACAATCC AAAATCGTAT CTACAAATTC	540
30	CGAGGTGATG GTAAGGCATG GGAATGGGAA GTAGATTGCG AAAATGGGAA TTATGATTAT	600
	TTAATGTATG CAGATGTAGA TATGGATCAT CCGGAGGTAG TAAATGAGCT TAGAAGATGG	660
	GGAGATGCT ATACAAATAC ATTAAATCTT GATGGAATTA GGATCGATGC GGTGAAGCAT	720
35	ATTAAATATA GCCTTACACG TGATTGGTTG ACCCATGTAA GAAACGCAAC GGGAAAGAGAA	780
	ATGTTTGCTG TTGCTGAATT TTGGAAAAAT GATTTAGGTG CCTGGAGAAA CTATTTAAT	840
40	AAACAAACT GGAATCATTC TGTCTTTGAT GTCCCGCTTC ATTATAATCT TTATAACGCG	900
	TCAAAATAGT GAGGCAACTA TGACATGGCA AAACCTCTTA ATGCAACGGT TGTTCAAAG	960
	CATCCAATGC ATGCCGTAAC TTTTGTGGAT AATCAGGATT CTCAACCTGG GGAATCATTA	1020
45	GAATCATTTG TACAAGRATG GTTTAAGCCA CTGCTTTATG CGCTTATTTT AACAAGAGAA	1080
	CAAGGCTATC CCTCTGTCTT CTATGGTGAC TACTATGGAA TTCCAACACA TAGTGCCCA	1140
50	GCAATGAAAG CCAAGATTGA TCCATCTTA GAGGCGCGTC AAAATTTTGC ATATGGAACA	1200
	CAACATGATT ATTTTGACCA TCATAATATA ATCGGATGGA CACGTGAAGG AAATACCCAG	1260
55	CATCCCAATT CAGGACTTGC GACTATCATG TCGGATGGGC CAGGGGGAGA GAAATGGATG	1320

TACGTAGGGC AAAATAAAGC AGGTCAASTT TGGCATGACA TAACTGGAAA TAAACCAGGA 1380

ACAATTACGA TCAATGCAGA TGGATGGGCT AATTTTTCAG TAAATGGAGG ATCTGTTTCC 1440

5 ATTTGGGTGA AACGA 1455

(2) INFORMATION FOR SEQ ID NO: 15:

(i) SEQUENCE CHARACTERISTICS:

- 10 (A) LENGTH: 13 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

- 15 (A) DESCRIPTION: /desc = "Primer BSG1"
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

CCAATGATGCA GTATTTTGAA TGG

13

20

(2) INFORMATION FOR SEQ ID NO: 16:

(i) SEQUENCE CHARACTERISTICS:

- 25 (A) LENGTH: 12 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

- 30 (A) DESCRIPTION: /desc = "Primer BSG1"
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 16:

GTCACCATAA AAGACGCACG GG

12

(2) INFORMATION FOR SEQ ID NO: 17:

(i) SEQUENCE CHARACTERISTICS:

- 35 (A) LENGTH: 70 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

40 (ii) MOLECULE TYPE: other nucleic acid

- (A) DESCRIPTION: /desc = "Primer BSG1"
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 17:

GTCATAGTTT CCGAATTCGG TGTCTACTTC CCAATCCCAA TCCCAAGCTT

45 TCCCGCGGAA TTGTAAATG

70

(2) INFORMATION FOR SEQ ID NO: 18:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 41 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
5 (D) TOPOLOGY: linear
 (ii) MOLECULE TYPE: other nucleic acid
 (A) DESCRIPTION: /desc = "Primer BSGM2"
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 18:

10 CTACTCCCA ATCCCAAGCT TTGCCGCGGA ATTTGTAAAT G

41

(2) INFORMATION FOR SEQ ID NO: 19:
 (i) SEQUENCE CHARACTERISTICS:
15 (A) LENGTH: 26 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear
 (ii) MOLECULE TYPE: other nucleic acid
20 (A) DESCRIPTION: /desc = "Primer BSGM3"
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 19:

GGATGATCCA TGTCAAAGTG GCATAC

26

25

(2) INFORMATION FOR SEQ ID NO: 20:
 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 25 base pairs
 (B) TYPE: nucleic acid
30 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear
 (ii) MOLECULE TYPE: other nucleic acid
 (A) DESCRIPTION: /desc = "Primer BSGM4"
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 20:

35

CTCGGTCACC ACGTGGGGAT GATCC

25

(2) INFORMATION FOR SEQ ID NO: 21:
40 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 24 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear
 (ii) MOLECULE TYPE: other nucleic acid
45 (A) DESCRIPTION: /desc = "Primer BSGM5"
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 21:

CCAGTTTTTC AGCTGGGTCA GGAC

INTERNATIONAL SEARCH REPORT

International application No.

PCT/DK 98/00444

A. CLASSIFICATION OF SUBJECT MATTER

IPC6: C12N 9/28, C11D 3/386

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: C12N, C11D

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPI, PAJ, BIOSIS, CA

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	WO 9741213 A1 (NOVO NORDISK A/S), 6 November 1997 (06.11.97), page 15, line 23 - page 17, line 4 --	1-33
X	WO 9623873 A1 (NOVO NORDISK A/S), 8 August 1996 (08.08.96), page 21 - page 38; page 75 - page 77 --	1-33
X	WO 9510603 A1 (NOVO NORDISK A/S), 20 April 1995 (20.04.95), page 18, line 1 - page 20, line 14 --	1-33
A	WO 9535382 A2 (GIST-BROCADES B.V.), 28 December 1995 (28.12.95), page 3, line 20 - line 26, claims --	1-33

☒ Further documents are listed in the continuation of Box C.☒ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"I" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"Z" document member of the same patent family

Date of the actual completion of the international search

20 January 1999

Date of mailing of the international search report

125 -01- 1999

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/DK 98/00444

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 9100353 A2 (GIST-BROCADES N.V.), 10 January 1991 (10.01.91) -- -----	1-33

INTERNATIONAL SEARCH REPORT

International application No.

PCT/DK 98/00444

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

The claimed inventions relates to variants of a parent Termamyl-like alpha-amylase.

A large number of combinations of mutations are suggested, which give increased thermostability at acid pH and/or low Ca^{2+} concentrations.

Several different combinations of mutations of α -amylases giving more thermostable

enzymes are well-known in the art, see search report. As no common theory for all the mutations are suggested in the present application no "special technical feature" that makes a contribution to the prior art, as demanded in PCT rule 13.2 has been found.

Although the application claims a large number of inventions all of them have been searched.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☒ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT
Information on patent family members

01/12/98

International application No.

PCT/DK 98/00444

Patent document cited in search report			Publication date	Patent family member(s)	Publication date
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